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CONTENTS · TABLE DES MATIÈRES · INHALT

Page

von Arx, J. A., Ein neuer Ascomycet aus Afrika 161

Bekker, J. H. and Mossel, D. A. A., Evaluation of a medium containing tergitol-7 and triphenyltetrazolium chloride for the differentation of coliform bacteria from drinking water and foods . . 252

Bekker, J. H. and Onvlee, P. C., Experiences with the treponemal immobilization test in the Netherlands 331

Bekker, J. H. s. Hylkema, B.

Bekker, J. H. s. Vink, H. H.

Bezjak, V., Differentation of *Corynebacterium diphtheriae* of the *mitis* type found in diphtheria and ozaena. II. The rate of rapidity of glucose fermentation by *C. diphtheriae* type *mitis* and *C. belfanti* 45

Bokkenheuser, V. and Richardson, N., Observations on the use of dried *Salmonella* antigens 239

Brandwijk, A. C. s. Tasman, A.

Bulder, C. J. E. A., Some observations on the lipolytic activity of micro-organisms and a new method for its detection 433

Buttiaux, R., Le difficile problème bactériologique des semi-conserves de viandes en boîtes 257

Capriotti, A., Yeasts in some Netherlands soils 145

Clarenburg, A. s. Keizer, D. P. R.

Clarenburg, A. s. Vink, H. H.

Collier, W. A. und Jacoeb, M., Bacterien-Haemagglutination. I. Versuche mit einem haemagglutinirenden Stamm von *E. coli* 113

Collier, W. A., Sue Tsiew Wong und de Miranda, J. C., Bacterien-Haemagglutination. II. Unspecifische Hemmung der Coli-Haemagglutination 124

Collier, W. A. und de Miranda, J. C., Bacterien-Haemagglutination. III. Die Hemmung der Coli-Haemagglutination durch Mannose . . . 133

Collier, W. A., Tiggelman-van Krugten, V. A. H. und Tjong A Hung, T., IV. Ueber Haemagglutinationsversuche bei 105 Colistämmen und Adsorption der Haemagglutinine 303

Deom, J. et Mortelmans, J., De la sensibilité *in vitro* de quelques micro-organismes d'origine animale à la viomycine 377

Deom, J. et Mortelmans, J., De la sensibilité *in vitro* de quelques micro-organismes d'origine animale à la neomycine 382

Devos, A. s. Geurden, L. M. G.

Dolman, H., Miss Ebels, I. and Winkler, K. C., Reproduction of bacteriophage T_6 and T_7 in auxotrophic mutants of *E. coli* B 369

Ebels, I. s. Dolman, H.

Edwards, P. R., Rutten, F. J. and McWhorter, A. C., Four new *Salmonella* serotypes isolated in Curaçao 80

Farber, S. s. Foley, G. E.

Foley, G. E. and Haley, E. C., Chemical and biological studies on 1,2-dihydro-*s*-triazines. VII. Activity in pteroylglutamic acid-*Lactobacillus casei* 7469 systems 385

Foley, G. E. and Haley, E. C., Chemical and biological studies on 1,2-dihydro-*s*-triazines. VIII. Activity in nicotinic acid, riboflavin and thiamine systems 397

Foley, G. E. and Haley, E. C., Chemical and biological studies on 1,2-dihydro-*s*-triazines. IX. Inhibition of synthesis of citrovorum factor in *Leuconostoc citrovorum* 8081 systems 405

64-00532

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CONTENTS TABLES DES MATIÈRES INHALT

Page

Foley, G. E., Modest, E. J., Farber, S. and Haley, E. C., Chemical and biological studies on 1,2-dihydro-*s*-triazines. X. Mechanism of action in microbiological systems 417

Geurden, L. M. G. and Devos, A., Investigations on some properties of *Listerella monocytogenes* 169

Gispen, R. s. Heyl, J. G.

Godin, P., Etude des premières étapes de la dégradation du glucose chez *Penicillium brevi-compactum* 83

Godin, P., Production de substances phénoliques aux dépens de divers composés ternaires par *Penicillium brevi-compactum*. 94

Godin, P., Etude du processus de transformation des acides di- et tricarboxyliques chez *Penicillium brevi-compactum* 177

Godin, P., Filiation biochimique des acides phénoliques produits par *Penicillium brevi-compactum* 215

Godin, P., Séparation, par chromatographie sur colonne de cellulose, des substances phénoliques produites par *Penicillium brevi-compactum* et analyse chimique sommaire de l'une d'entre elles 362

de Haan, P. G. and Winkler, K. C., An apparatus for the continuous culture of bacteria at constant generation times. 33

de Haan, P. G., and Winkler, K. C., Phage reproduction in relation to bacterial growth rate 103

Haley, E. C. s. Foley, G. E.

Heyl, J. G. and Gispen, R., A complement fixing toxoplasma antigen prepared from duck eggs and enhanced by phenol 157

Houwink, A. L., *Caulobacter*. Its morphogenesis, taxonomy and parasitism 49

Hung, T. Tjong A. s. Collier, W. A.

Hylkema, B. and Bekker, J. H., A roll-tube technic for the study by fluorescence-microscopy of the growth of Mycobacteria 28

Hylkema, B., An investigation into a suitable medium for the cultivation of *Mycobacterium tuberculosis* from pathological material in roll-tubes 289

Jacoeb, M. s. Collier, W. A.

Keizer, D. P. R., Clarenburg, A. and Vink, H. H., Some remarks on *Salmonella paratyphi* type Heidelberg 141

McWhorter, A. C. s. Edwards, P. R.

Minkenhof, J. E. s. Tasman, A.

de Miranda, J. C. s. Collier, W. A.

Modest, E. J. s. Foley, G. E.

Mohan, R. R. s. Schatz, A.

Mortelmans, J. s. Deom, J.

Mossel, D. A. A. and Tollenaar, F. D., A simple test for use in experiments on the occurrence and the prevention of microbial lipolytic spoilage in foods 247

Mossel, D. A. A. s. Bekker, J. H.

de Nooy, J. A. s. Vink, H. H.

Onvlee, P. C. s. Bekker, J. H.

van Ramshorst, J. D. and Rijks, H., The flocculation of tetanus toxin and toxoid. II. 186

Reiersøl, S., Species of *Rhodotorula* isolated from laryngeal swabs . . 286

Richard, O. s. Wikén, T.

Richardson, N. s. Bokkenheuser, V.

CONTENTS TABLE DES MATIÈRES INHALT

Page

la Rivière, J. W. M., The production of surface active compounds by micro-organisms and its possible significance in oil recovery. I. Some general observations on the change of surface tension in microbial cultures 1
la Rivière, J. W. M., The production of surface active compounds by micro-organisms and its possible significance in oil recovery. II. On the release of oil from oil-sand mixtures with the aid of sulphate reducing bacteria 9
Rutten, F. J. s. Edwards, P. R.
Rijks, H. s. van Ramshorst, J. A.
Schatz, A., Mohan, R. R. and Trelawny, G. S., Oxidation of tricarboxylic acid cycle intermediates by *Streptomyces nitrificans* 225
Schönfeld, J. K., Investigations into the combined action of penicillin and streptomycin in vitro 316
Smith, L. s. Tasman, A.
Stolk, A. C., The genera *Anixiopsis* Hansen and *Pseudeurotium* van Beyma 65
Sue Tsiew Wong s. Collier, W. A.
Tasman, A., Minkenhof, J. E., Brandwijk, A. C. and Smith, L. On the pathogenesis of diphtheria. IV. On the occurrence of diphtheria toxin in the saliva of diphtheria patients 193
Tiggelman-van Krugten, V. A. H. s. Collier, W. A.
Tollenaar, F. D. s. Mossel, D. A. A.
Trelawny, G. S. s. Schatz. A.
Vago, C. et Vasiljevic, L., Une virose à localisation intestinale avec affinité cytoplasmique chez le Lépidoptere *Thaumatopoea processionea* 210
Vasiljevic, L s. Vago, C.
Vink, H. H., Clarenburg, A., de Nooy, J. A. and Bekker, J. H., A new *Salmonella* type (*S. haarlem*) 367
Vink, H. H., Spontaneous tuberculosis in the guinea pig 446
Vink, H. H. s. Keizer, D. P. R.
de Vries, G. A., *Cladosporium avellaneum* de Vries, a synonym of „*Hormodendrum*" *resinae* Lindau 166
Westendorp Boerma, F., Human O erythrocytes in the hemagglutination reaction for rheumatoid arthritis 277
Wikén, T. und Richard, O., Untersuchungen über die Physiologie der Weinhefen. VI. Mitteilung. Weitere Untersuchungen über die Bedeutung der Stickstoff- bzw. Argonbegasung und des Zusatzes kleiner Sauerstoffmengen für die Vergärung der Glucose durch die Weinhefe „Fendant" aus Kulturen verschiedenen Alters. Vergleich mit der Untergärigen Bierhefe „Müllerbräu" und der Brennereihefe „Taranto" 337
Winkler, K. C. s. de Haan, P. G.
Winkler, K. C. s. Dolman, H.

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THE PRODUCTION OF SURFACE ACTIVE COMPOUNDS BY MICRO-ORGANISMS AND ITS POSSIBLE SIGNIFICANCE IN OIL RECOVERY

I. SOME GENERAL OBSERVATIONS ON THE CHANGE OF SURFACE TENSION IN MICROBIAL CULTURES

by

J. W. M. LA RIVIÈRE

(Received March 12, 1954).

1. INTRODUCTION.

Many surface active agents show in comparatively low concentrations a marked effect on the development of micro-organisms. These chemically so divergent compounds, characterized by their common physico-chemical behaviour show so much similarity in their action on living cells that several investigators consider them as a separate class of agents, also from a bacteriological point of view (LASSEUR *et al.*, 1932, GLASSMAN, 1948).

In the earlier literature (e.g. FROBISHER, 1926) their activity was ascribed without much explanation to the depression of surface tension brought about in the culture medium, and by some authors surface tension was given the status of "milieu-factor" along with pH, temperature etc. This view has since been abandoned for several reasons. In the first place a measured surface tension depression of the medium has no quantitative relationship to the concentration of the active compound at the interface between cell and medium. Moreover it has been found that, when an accumulation at this interface does take place, the chemical nature of the concentrated substance plays a dominant role in the observed phenomena, as has been amply demonstrated for example in toxicity experiments (MITCHELL, 1951).

In those cases, however, where mainly the distribution of the cells in liquid and solid media is found to be affected, such as pellicle formation versus diffuse growth (LARSON *et al.*, 1919, DUBOS and DAVIS, 1946), films on glass (ZOBELL, 1943), colony morphology (ROOK and BRUCKMAN, 1953), it is difficult to deny an important influence to the purely physical alterations in the medium, *i.e.* the lowered surface and interfacial tensions. The work of MUDD and MUDD (1924) on the influence of interfacial tension on the distribution of particles in a system of two liquids, as applied to bacteria, is most illuminating. Also localisation of growth in interfaces may be quite naturally expected in cases where essential nutrients, present in low overall concentrations, are surface active.

We may, therefore, conclude that since a long time the importance of surface active compounds in bacteriology has been recognized. This has even led to a wide application of these compounds in bacteriological practice, such as in Dubos' medium for the cultivation of *M. tuberculosis*, Mac Conkey broth, desinfection etc.

It is, however, striking that, where so much work has been done on the effect of surface active compounds added to the culture medium, one finds in the literature only very few reports on the production of surface active substances by the bacteria themselves.

The few data available are the results of measurements in ill-defined, non-synthetic media, which already contained surface active nutrients (WOLF, 1923, KOPACZEWSKI, 1931, REID, 1935, ZOBELL, 1947). The results obtained by these authors may, therefore, be due to more than one process: decrease of the concentration of some surface active nutrient, formation of surface active breakdown products of some component of the substrate, or finally the synthesis of surface active matter in the bacterial cell.

In the course of an investigation on the release of oil from oil-sand mixtures under the influence of sulphate reducing bacteria (Part II), it became desirable to make some surface tension measurements on cultures of these bacteria. It was then decided to extend these observations to other organisms in order to obtain a more general insight into the changes of surface tension of cultures of micro-organisms during growth.

As the interaction of different surface active agents is difficult to estimate (NIVEN, 1950), it offers many advantages to start with the study of mineral media which only contain one organic nutrient without surface activity.

It should be taken into account that certain dissimilatory processes will give rise to a production of surface active substances in relatively large quantities, *e.g.* those in which ethyl alcohol or butyric acid are formed from glucose, or those which lead to a simple hydrolysis of esters of fatty acids. We have, therefore, centred our attention at those cases in which such an effect could not be foreseen.

In the following the results of our measurements of changes in surface tension, occurring in bacterial cultures in synthetic media, will be reported.

2. METHODS.

Surface tension measurements were carried out by means of the Du Noüy tensiometer at room temperature (18°-22° C.). 10 ml of the culture liquid was brought in a small Petri dish with a diameter of 6.5 cm, which had been cleaned with chromo-sulfuric acid and rinsed with water. In most cases it was found necessary to allow a period of 30-60 minutes in order to attain equilibrium as manifested by the fact that on further standing no change of surface tension occurred. The migration of surface active matter to the surface appeared to be reversible, as shaking of the culture medium in the Petri dish resulted again in an increased surface tension, which on standing again fell back to the earlier observed minimum value after the same period of time. This phenomenon is not at all uncommon, and is often encountered in solutions of well defined surface active compounds (NIVEN, 1950).

Accordingly all measurements were made after 1 hour standing. All organisms — with the exception of the sulphate reducing bacteria — were grown in a basal medium of the following composition: tap water, 0.1 % NH_4Cl, 0.05 % K_2HPO_4, 0.05 % $MgSO_4$, trace $FeCl_3$, to which carbon sources were added, as indicated in Table II. The pH proved to be 7.0. The pH in the media for *Aspergillus niger, Candida lipolytica* and *Acetobacter aceti* was adjusted to 5.0.

Desulfovibrio desulfuricans, strain El Agheila Z, kindly put at our disposal by Dr K. R. BUTLIN, Chemical Research Laboratory, Teddington (England), was cultivated in the following medium: tap water, 0.1 % NH_4Cl, 0.2 % $MgSO_4$, 1.8 % Na_2SO_4 (anh.), 0.05 % K_2HPO_4, 1.0 % NaCl, 2.1 % Na-lactate; the pH was adjusted to 7.0. Immediately before inoculation this medium was freed from oxygen by boiling, followed by rapid cooling.

The sulphate reducing bacteria were cultivated in glass-stoppered bottles of 60 ml. The other organisms were cultivated under aerobic conditions in 50 ml flasks, containing 25 ml of the medium. Incubation took place at 30° C.; no shaking was applied.

All glassware had been cleaned with hot chromo-sulfuric acid before use. In order to obtain a representative sample for surface tension measurements 10 ml of the culture liquid were pipetted off after vigorous shaking, because surface active substances might have been concentrated in the surface. All measurements were carried out in duplicate; each time two 25 ml cultures of the same organism were tested.

3. RESULTS.

In Table I literature data (HODGMAN, 1948) on the surface tension of aqueous solutions of some organic compounds are compared with values obtained for a selection of some complex routine culture media.

TABLE I.

Surface tension of synthetic media and various aqeous solutions.

Aqeous solution	Surface tension in dyne/cm	Liquid	Surface tension in dyne/cm
1 % glycerol	72	water	72
1 % glucose	72	1 % peptone-water	54
1 % acetic acid	68	malt extract	47
1 % ethyl alcohol	66	yeast extract	47
1 % butyric acid	56	yeast autolysate	38
suspension of 5 g yeast in 1 l water	71	Mac-Conkey broth	39

In agreement with the observations of WOLF (1923) and MARSHALL (1924) all of these complex media showed a marked depression of the surface tension, as compared with that of pure water. In the concentration tested butyric acid is also clearly surface active, in contrast to what holds for glucose, glycerol and acetic acid. That the presence of a large number of cells, as will result from growth, does not in itself necessarily lead to a depression, is shown by the value obtained for the yeast suspension.

In Table II surface tension values are given for cultures of the micro-organisms as listed.

TABLE II.

Surface tension of cultures of various micro-organisms in synthetic media containing one single organic compound.

Organism	Mineral medium with	Surface tension in dyne/cm								Drop of surface tension in dyne/cm in 7 days
		Medium immediately after inoculation		1 day old cultures		3 days old cultures		7 days old cultures		
Bacillus subtilis	1 % glycerol	71	68	71	71	52	52	35	36	34
Aspergillus niger	1 % glycerol	67	68	71	70	66	68	51	53	15
Pseudomonas aeruginosa	1 % glycerol	70	71	71	71	59	59	49	49	21
Candida lipolytica	1 % glycerol	71	66	63	68	50	52	50	50	19
Desulfovibrio desulfuricans	2.1% Na-lactate	70	71	71	71	69	70	50	49	21
Aerobacter aerogenes	1 % glucose	65	68	68	71	63	60	52	53	14
Mycobacterium phlei	1 % glucose	71	71	70	65	58	57	54	58	15
Acetobacter aceti	1 % Na-acetate	70	70	68	69	59	61	56	56	14

Inoculation was as a rule carried out with one drop of a suspension of young agar slant cultures in water. With the sulphate reducing bacteria larger inocula had to be used; an inoculum of approximately 5 % was applied.

Apparently in no case a significant amount of surface active matter was carried over with the inoculum. Also after 1 day cultivation no important changes could be observed. After 3 days, however, the surface tension of most cultures had gone down, and after 7 days a definite drop in surface tension was noted in all cases. For *Bacillus subtilis* and *Pseudomonas aeruginosa* some additional values were determined as is shown in Fig. 1; the surface tension of the cultures of *Desulfovibrio desulfuricans* was followed over a longer period (Fig. 2).

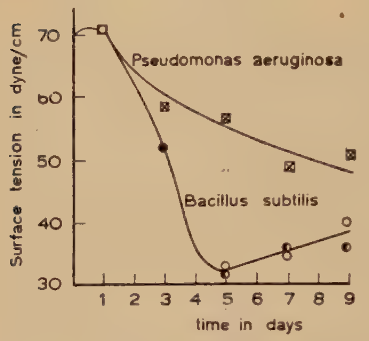

Fig. 1. Change of surface tension in cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* in a synthetic medium with glycerol as sole organic compound.

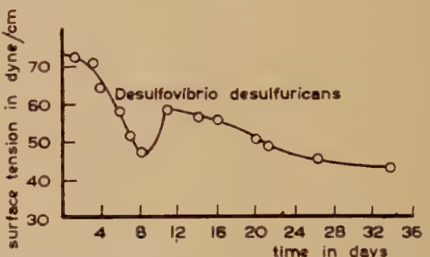

Fig. 2. Change of surface tension in cultures of *Desulfovibrio desulfuricans* in a synthetic medium with lactate as sole organic compound.

4. DISCUSSION.

The media used in these experiments cannot have been optimal for all organisms tested, and therefore rate and amount of growth have not been the same for the different organisms. Moreover, it is well known that there exists no linear relationship between surface tension and concentration of surface active matter. For this reason an attempt to a quantitative interpretation of the behaviour of the organisms would be of little value, and so we shall confine ourselves to a discussion of the qualitative significance of the results obtained.

The most striking result is perhaps that all organisms tested, although widely diverging in properties, have been found to produce surface active material, albeit to a varying extent. Especially the low values obtained in the cultures of *Bacillus subtilis* within a relatively short period are quite noteworthy.

Breakdown products of living material often show surface activity — which may be the explanation of the low surface tensions of the non-synthetic media — and, therefore, it is quite natural to expect that in the course of time any culture of a micro-organism, left to itself, will eventually contain some surface active matter due to autolysis of the cells.

That autolysis may, indeed, be one of the sources of surface active matter is supported by the observation that after a period of 3-4 weeks cultures of *Desulfovibrio desulfuricans* show a strongly reduced turbidity and that at the same time they have reached their final low surface tension values. In these old cultures only débris could be detected under the microscope.

On the other hand also active cells may well excrete small amounts of specific surface active substances. One needs only think of the excretion by certain cells of small quantities of enzymes, pigments, antibiotics etc. Isolation and identification of this type of compounds has in general only been possible because they show already in low concentrations in some way a strong activity. Thus it is very likely that other compounds, excreted in small quantities, will only have escaped detection, because they do not possess such easily observable properties. As the establishment of a depression in surface tension is definite proof for a change in composition of the liquid in question, a critical evaluation of surface tension changes as studied here, might be found to be of help in detecting unknown surface active substances.

Now that the production of surface active substances by bacterial cultures has been definitively established, further investigations should deal with the question if, and to what extent, these compounds influence the development of the culture.

The depression found in the cultures of *Desulfovibrio desulfuricans* confirms the results obtained by ZOBELL, whose observations, however, relate to media containing peptone. It has now been demonstrated that no such complex nutrient is necessary to obtain capillary changes in the medium with the aid of sulphate reducing bacteria. This result seems of importance in view of a possible application of these bacteria in oil recovery (LA RIVIÈRE, 1955).

Summary.

Cultures of 8 micro-organisms, grown in synthetic media free from surface active nutrients, were found to show a marked decrease in surface tension on prolonged incubation. The depression started after about two days cultivation and attained values ranging from 14—34 dyne/cm after 7 days. The possible origin of the substances responsible for this effect is briefly discussed.

Acknowledgement.

The investigation has been made possible by a grant from the "Stichting Molengraaff-fonds" to which the author wishes to express his sincerest thanks.

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(Laboratory of Microbiology, Technological University, Delft, Holland).

THE PRODUCTION OF SURFACE ACTIVE COMPOUNDS BY MICRO-ORGANISMS AND ITS POSSIBLE SIGNIFICANCE IN OIL RECOVERY

II. ON THE RELEASE OF OIL FROM OIL-SAND MIXTURES WITH THE AID OF SULPHATE REDUCING BACTERIA

by

J. W. M. LA RIVIÈRE

(Received March 12, 1954).

1. Introduction.

With present oil-production methods a recovery efficiency of roughly 40-60 % (Muskat, 1953) is obtained, and during the last decades much research has been done to find suitable secondary recovery methods in order to bring to the surface the vast amounts of oil that have been left behind in the reservoirs.

A simplified representation of an oil reservoir of a common type is given in Fig. 1.

Due to folding of the formation an oil trap has been formed in the oil and water bearing, porous, sand layer A. Owing to its lower

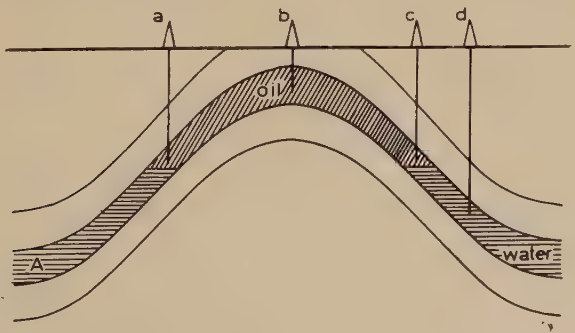

Fig. 1. A: oil and water bearing, porous sand layer. a, b, c and d: wells. For explanation see text.

specific weight the oil has accumulated at the top of the layer. When oil is withdrawn through wells *a*, *b* and *c*, the oil-water interface will rise and eventually only water will be produced by wells *a* and *c*. The thus freshly water-flooded section of the oil sand still contains considerable amounts of residual oil, part of which may be recovered by injection of suitable liquids or gases through well *d* and subsequently through wells *a* and *c*.

Injection of water, chemicals or gases (MUSKAT, 1949) has been found successful to a certain degree in many cases, but the problem is far from solved; MUSKAT (1953) points out that a rise of 1 % in recovery efficiency would increase the value of present known oil reserves in the U.S.A. with 1.4 billion dollars. It is therefore easily understood that in the hunt for new methods every promising possibility is eagerly explored.

ZOBELL (1946) was the first to draw attention to the possible usefulness of sulphate reducing bacteria and obtained a patent for a secondary recovery method in which the necessary measures to cultivate these bacteria in the water phase, present in the oil reservoirs, are described.

Since the publication (1947*a*) of his experiments underlying the proposed method only very few additional data have been published [1]). BECK (1947) studied on laboratory scale the possible application in the Bradford oil reservoir, but was not able, despite the great number of experiments undertaken, to reproduce ZOBELL's results. It seems that, notwithstanding words of warning of ZOBELL (1947*b*), unfounded optimistic reactions have been created among oil-companies (DEEGAN, 1947) which have resulted in a more or less sensational atmosphere which may have reflected unfavourably on the ultimate progress of the experimental work. As far as is known, no field tests have as yet been made and in consequence the practical usefulness of the method must still be considered to be doubtful (RÜHL, 1952). On the other hand no adequate experimental evidence is available disproving ZOBELL's claims, and now and then indications are found in literature that further research has not yet been abandoned (anonymous publication: Ind. Eng. Chem. **44**, 15A, 1952; MACKENZIE, 1952; SCHWARZ, 1953).

Now it must at once be stated that the problem is by no means a simple one, as has already been emphasized by ZOBELL. It may well be that reproduction of good results obtained under laboratory conditions in a certain number or even all types of reservoirs will prove to be impossible, but before a decision can be reached, a full understanding of the mechanism and the scope of oil-release by bacteria as can be obtained in laboratory experiments must be acquired. The present study aims in the first place at a reproduction of some observations reported by ZoBell, and secondly at making a contribution towards further insight into the responsible mechanism.

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[1]) Cf. also the survey in the recently published book of BEERSTECHER (1954), Petroleum Microbiology. (*note added in proof*).

2. A SURVEY OF ZOBELL'S PUBLICATIONS.

The basic phenomenon of oil release was discovered by ZoBell (1947) during experiments on the anaerobic consumption of hydrocarbons by sulphate reducing bacteria. A greater contact area between oil and water phase was obtained by a method already used by Baars (1930): a mixture of oil and sand was brought on the bottom of glass-stoppered bottles fully filled with the aqueous medium for the bacteria. In those bottles in which after inoculation and incubation growth of bacteria occurred, ZoBell regularly observed release of oil from the sand, resulting in an accumulation of small amounts of oil in the top of the bottle. In bottles which did not show a marked growth no release was observed. The phenomenon did not depend on the kind of medium used, provided growth took place. Consumption of hydrocarbons, as sole source of carbon, was therefore no essential condition for oil release.

These observations led ZoBell to a consideration of the mechanisms by means of which bacteria in general may release oil. His ideas can be roughly summarized as follows:

1. Attack on the solid phase by acidification, carbonate or sulphate reduction.
2. Expulsion of oil by production of gas, either by the mechanical effect of formed bubbles, or if at high pressures in the reservoir the produced gas dissolves in the oil, by lowering the oil viscosity.
3. Production of surface active substances.

A further study of the last mechanism seemed the most promising for the following reasons:

ad 1). Many reservoir formations consist of sand stone which in contrast to carbonate or sulphate rock cannot be attacked by bacteria. Moreover injection of acid solutions is already done with success and it is difficult to see that the use of bacteria would offer advantages. Application would thus be restricted to the rarely occurring sulphate rock reservoirs, where sulphate reduction might be effective in corroding the solid phase.

ad 2). Gas injection is already a known practice in oil production; bacterial gas production might be more effective as locally small bubbles may be produced. However, the high pressure in the reservoir will as a rule lead to the solution of the gas in oil or water phase, so that the advantage of bacterial gas production above gas injection seems rather questionable.

ad 3). It is true that a direct injection of solutions of synthetic surface active agents into the formations could be tried and laboratory experiments have yielded promising results (MUSKAT, 1949). Field practice has proved, however, to be unsuccessful, because by their polar nature these compounds are readily adsorbed at the surface in the formation, and as a consequence do not penetrate into the reservoir much further than the point of injection.

It is here that the chances for an essential contribution by bacteria seem most favourable. Their action in this respect will be indepenpendent of the nature of the rock, and it is conceivable that solutions carrying small amounts of non polar not easily adsorbed nutrients can permeate the whole formation and that subsequent bacterial growth will result in a production of surface active agents *in situ*.

ZOBELL's suggestion that surface tension is an active principle in oil release was put forward in order to explain the at first sight mysterious oil release in the experiments outlined above. Gas and acid production are not pronounced in cultures of sulphate reducing bacteria and as sand was used in the experiments, action of sulphate reduction on the solid phase has to be excluded. As another explanation ZOBELL mentions the tendency of these bacteria to grow in films on solid surfaces; release might be effected by crowding off the oil by growth of the film-forming cells on the surface of the sand particles. However, ZOBELL himself found that old cultures showed oil releasing activity even after sterilization had taken place. As a consequence any mechanism involving direct action of living cells on either oil or sand is at most of secondary importance.

To substantiate his hypothesis ZOBELL measured the surface tension of sterile medium and of a culture after growth of 6 weeks. He regularly found a pressure drop, in one case from 63 dyne/cm to

46 dyne/cm. As the media tested, as far as specified in his paper, contained 1 % peptone, which in itself shows surface activity, the possibility cannot be excluded that the compounds responsible for the observed depression originate from the peptone and have been liberated from it, *e.g.* by simple hydrolysis. In other words, the compounds in question may not be synthetic products of the bacteria, and the pressure drop observed might be largely dependent on the nature of the culture medium used.

The literature on production of surface active substances by bacteria in general is very scanty and seemed to justify the special study reported in Part I (LA RIVIÈRE, 1955). There it was found that a number of organisms, including sulphate reducing bacteria, are indeed able to bring about a considerable reduction of surface tension on cultivation in synthetic media free from surface active nutrients. It should be noted that ZOBELL did not investigate whether under the conditions of his experiments addition of synthetic surface active material led to a comparable oil release as obtained by bacterial growth.

Moreover, a further study of the principles of secondary recovery has made it clear that more than surface tension, interfacial tension between oil and water phases is to be regarded as a measure for oil liberating qualities of aqueous solutions (MUSKAT, 1953).

Taking the foregoing into consideration, it was decided to repeat the experiments of ZOBELL with media that did not contain surface active nutrients, and to measure its surface and interfacial tension before and after growth had taken place. Furthermore experiments were carried out to compare the bacterial activity with that of an addition of a synthetic detergent. Finally a method was sought to arrive at a better evaluation of the oil releasing power of the liquids in question than was possible with the bottle method.

3. EXPERIMENTAL METHODS.

a. Surface- and interfacial tension measurements.

These were carried out with the du Noüy tensiometer. Interfacial tension was measured against a clear kerosene sample. One hour standing before measurement proved adequate for equilibration (Cf. Part I).

b. Bacteria, media, cultivation.

The strain of *Desulfovibrio desulfuricans* El Agheila Z was used in all experiments. It was kept in an active state by weekly transfers in the liquid medium of MILLER (1949) in stoppered bottles (Part I). No $CaCO_3$ was added in order to restrict the solid phase to sand; iron was also left out so as to facilitate observation on oil displacements. In this simplified medium normal growth was found to take place; 2-3 days after inoculation the medium had become distinctly turbid. Large inocula of about 10 % were used in order to obtain a quick response; for the same reason immediately before use oxygen was removed from the medium by boiling, followed by rapid cooling to the incubation temperature of 30° C.

c. Oil-sand mixtures.

Natural oils were kindly put at our disposal by the Bataafsche Petroleum Maatschappij and the Nederlandse Aardolie Maatschappij. Experiments were carried out with Trinidad oil and oil from the Dutch reservoir at Schoonebeek. Oil-sand mixtures were made up with air-dry river sand or non-consolidated sand from the Schoonebeek formation by thoroughly mixing in a mortar. The oil percentage in the mixture varied from 9—16 %.

d. Estimation of the amount of oil released in bottle experiments.

The amount of oil liberated from oil-sand mixtures by different aqueous phases was determined by dissolving the layer of supernatant oil in ether. The solution was pipetted off carefully, and brought in a flat porcelain dish of known weight. The surface of the water phase was repeatedly washed with ether until all oil was removed; the oil adhering to the stop of the bottle was collected in the same way.

The greater part of the ether was allowed to evaporate; the last traces were removed by keeping the dishes for 1 hour in an evacuated desiccator containing silica-gel, as the ether had become moist by contact with the waterphase in the bottle. The amount of oil was finally determined by weighing the dish. In control tests the error was found to be within 5% of the amount of oil determined.

e. Estimation of oil release promotion in a glass bead column.

The fluid to be tested on its oil-releasing properties was pressed by its own weight through a vertical column of glass beads (Fig. 2).

The beads had a diameter of 2 mm. The column was 11 cm high; its diameter measured 12 cm. The upside end of the tube containing the beads, was narrowed and calibrated in order to make measurement of released oil possible. The fluid was kept in circulation through an outlet on the side of the column above the glass-beads. In a reservoir 20 ml of oil was kept ready; introduction of the oil took place after the whole column was filled with the aqueous liquid under investigation, by closing stopcock A and opening B and C.

The liquid passed through the column was caught in a measuring cylinder and poured back in 1 l portions into the pressure reservoir. The average volume of liquid pressed through varied between 3 and 4 l/hr. The level of the aqueous phase in the pressure reservoir fluctuated from 12-2 cm above the outlet of the glass bead column. The fluctuating flow obtained by this procedure was reproduced exactly in every experiment and gave consistent results in duplicate tests. The amount of liquid pressed through the column at the moment when the first oil was released was taken as a measure for the oil releasing properties of the liquid. After each experiment the glass beads were cleaned by vigorous rinsing under a spout of tap water followed by washing in resp. petroleum ether, tap water, chromo-sulfuric acid and tap water. All tests were carried out at room temperature, and only Trinidad oil was used.

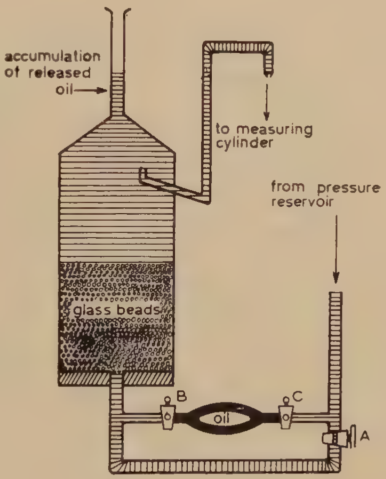

Fig. 2. Glass bead column for estimation of the oil releasing ability of a flowing aqeous phase.

It is realized that the simple set-up as outlined above can be improved in several ways. However, for the limited scope of the present experiments it proved to be satisfactory.

4. EXPERIMENTAL RESULTS.

a. Bottle experiments on oil release.

On the bottom of glass stoppered bottles oil-sand mixtures were brought as indicated in Table I. Sterilization of these mixtures proved to be very difficult; in dry sterilization low boiling oil components were lost by evaporation and in wet steam sterilization oil release occurred spontaneously. Therefore we resorted to the

TABLE I.

Amount of oil released in bottle experiments after 3 weeks incubation at 30° C. Medium: tap water, 0.1 % NH_4Cl, 0.2 % $MgSO_4$, 1.8 % Na_2SO_4 (anh.), 0.05 % K_2HPO_4, 1.0 % NaCl, 2.1 % Na-lactate.

Experiment No	Oil-sand mixture	% oil in mixture	Number of experiments	Aqueous phase	Amount of oil released		
					in g	Average amount in g	Average amount in % of oil present
1	50 g river sand + 5 g Trinidad oil	10	2	medium + 0.1% $HgCl_2$	0 0	0	0
			2	inoculated medium	0.181 0.785	0.483	9.6
			2	medium + 0.1% $HgCl_2$ + 0.1% Tween 20	0.438 2.008	1.223	24.4
2	55 g Schoonebeek sand +7.5 g Schoonebeek oil	13.6	1	medium + 0.1% $HgCl_2$	0	0	0
			1	inoculated medium	1.970	1.970	26.2
3*	50g Schoonebeek sand +4.5 Schoonebeek oil	9	1	medium + 0.1% $HgCl_2$	0	0	0
			1	inoculated medium	1.574	1.574	35
4	40 g river sand + 4 g Schoonebeek oil	10	6	medium + 0.1% $HgCl_2$	no oil released	0	0
			6	inoculated medium	no oil released	0	0
			3	medium + 0.1% $HgCl_2$ + 0.1% Tween 20	0.149 0.273 0.207	0.209	5.2
5	50 g river sand + 6.5 g Schoonebeek oil	13	6	medium + 0.1% $HgCl_2$	0.355 0.509 0.201 0.354 0.266 0.216	0.316	4.8
			6	inoculated medium	0.682 0.626 0.535 0.877 0.706 0.664	0.681	10.4
			3	medium + 0.1% $HgCl_2$ + 0.1% Tween 20	1.045 0.783 0.769	0.865	13.2
6	50 g river sand + 8 g Schoonebeek oil	16	6	medium + 0.1% $HgCl_2$	1.161 1.097 1.079 1.016 1.010 0.902	1.044	13
			6	inoculated medium	2.606 2.038 1.960 1.834 1.268 1.222	1.821	22.7
			3	medium + 0.1% $HgCl_2$ + 0.1% Tween 20	3.800 3.044 6.350	4.398	54.9

* In experiment No 3 a somewhat different medium and another bacterial strain were used.

use of a large amount of inoculation material (10–30 %). The salinity of the medium and the rather high initial H_2S content apparently kept growth of contaminating organisms within reasonable bounds, as regular microscopic observation did not show impurities and strong H_2S formation took always place. The bottles were entirely filled with the inoculated medium, closed and incubated at 30° C.

Control bottles containing sterile medium with 0.1 % $HgCl_2$ were also placed at 30 °C.

To compare the effect of a synthetic detergent 0.1 % Tween 20 was added to some of the control bottles.

Within 24 hours oil release could be observed in the bottles with Tween solution, whilst the bacterial cultures after 2—3 days began to show oil liberation. After 3 weeks the amount of oil released in each bottle was determined (Table I). At that time a strong production of H_2S had taken place; in 20 of the 22 bacterial cultures the H_2S content was determined iodometrically and found to be about 1 g/l.

In 16 out of 22 bottles in which bacteria had grown, and in all 11 bottles which contained Tween 20, more oil was released than in the corresponding control bottles.

The 6 bottles, in which bacteria did not release oil at all, belonged to the same series of experiments (no. 4). Apparently the oil content of the mixture was too low; in the experiments no. 5 and 6, in which the oil-sand mixture contained more oil, a definitely increased release under the influence of the bacteria was always noted.

The net result of the action of bacteria and of Tween 20, *i.e.* the excess of oil liberated in these cases over the amount obtained in the control bottles, is listed in Table II.

As the oil-sand mixtures used in experiments 1 and 4 differ in type of oil added only, a comparison can be made between both oils. Apparently Trinidad oil is better released by bacteria than the more viscous Schoonebeek oil.

Also the type of sand seems to be of importance, since Schoonebeek oil is much better released from Schoonebeek sand than from river sand (experiments no 2 and 5).

Qualitatively Tween solutions showed the same effect as the bacterial cultures did, but the amounts of oil released are higher.

From the 3 experiments with river sand and Schoonebeek oil (no 4, 5 and 6) it can be concluded that a higher amount of oil in the initial mixture leads to more release, as well by the bacteria as

TABLE II.

(Summary)

Amount of oil released in bottle experiments by bacteria and Tween 20 in excess of quantity released in corresponding control.

Experiment No	Oil-sand mixture	% oil in oil-sand mixture	Oil released in %, due to action of	
			bacteria	Tween 20
1	river sand + Trinidad oil	10	9.6	24.4
2	Schoonebeek sand + Schoonebeek oil	13.6	26.2	—
3*	Schoonebeek sand + Schoonebeek oil.	9	35	—
4	river sand + Schoonebeek oil	10	0	5.2
5	,, ,,	13	5.6	8.4
6	,, ,,	16	9.7	41.9

* In experiment No 3 a somewhat different medium and another bacterial strain were used.

by the Tween solution. Obviously a greater fraction of the oil is liberated when the oil concentration in the mixture is raised.

Although these experiments clearly demonstrate a positive effect of bacteria in oil release we are still far from knowing whether the observed facts are anything more than a laboratory curiosity. The important question is whether in a reservoir in operation the flowing aqueous phase will liberate more oil when sulphate reducing bacteria have been grown in it. It might well be that in the bottle experiments an amount of loosely held oil has been liberated which certainly would have been included in the quantity of oil set free when a normal oil recovery method had been applied on the oil-sand mixtures in question.

In order to check this, it was decided to carry out experiments in which the oil releasing effect of slow currents of the same liquids as were used in the bottle experiments could be studied.

b. Estimation of oil releasing properties in a glass bead column.

Some characteristics of the liquids tested are given in Table III.

TABLE III.

Interfacial- and surface tensions of liquids tested on oil releasing properties in glass bead column.

Liquid	Interfacial tension against kerosene in dyne/cm	Surface tension in dyne/cm
Lactate medium	27—33	69—70
Cultures of *Desulfovibrio desulfuricans*, more than 4 weeks old	12—19	44—46
Lactate medium + 0.1 % Tween 20	5	36
Sewage	—	44
Sewage + 2 % Na_2SO_4, 30 days cultivated at 30° C.	—	43

Encouraged by positive results of qualitative bottle tests, it was decided to include in these experiments some sewage samples.

The procedure followed was the same for all liquids. The results are given in Table IV.

It is evident that although there is some variation in the value for one and the same liquid, significant differences are found between on the one hand the sterile medium, and on the other hand the culture medium after growth, and finally the medium to which Tween had been added. The two latter liquids release the oil after about 4 l have been pressed through, *i.e.* 3 times as rapidly as the sterile medium does.

The fresh sewage already had a low surface tension. The results obtained for the sewage to which 2 % sodium sulphate had been added, and which had been incubated for 30 days at 30° C., are distinctly more favourable than those of the non-incubated control, to which 2 % NaCl had been added in order to compensate for

TABLE IV.

Estimation of the oil releasing ability of a flowing aqueous phase with the aid of the glass bead column.

Streaming aqueous phase	Average velocity of aqueous phase in 1/hr	Amount of aqueous phase in 1 pressed through at moment when first oil is released	
Lactate medium	3.4	13.75	average: 12.4
	3	12.75	
	2.9	13	
	4	10	
Cultures of *Desulfovibrio desulfuricans*, more than 4 weeks old	3.2	4	average: 4.1
	3.8	4.5	
	3.4	4	
	3.5	4	
Lactate medium + 0.1% Tween 20	3.1	3	average: 4.5
	3	7	
	3.8	5	
	3	3	
Sewage + 2% Na_2SO_4, 30 days cultivated at 30° C.	3.1	5.4	average: 5.2
	3	5	
Sewage + 2% NaCl, kept at 5° C. for 30 days	3.5	8.2	average: 8.3
	3.3	8.4	

the difference in specific weight. Comparison of the sewage samples with the other liquids is not admissible, as the latter have a higher specific weight,

The bottle experiments had already definitely shown that there existed an effect due to bacterial growth, but the question was left open whether the improvement of oil releasing properties effected by the bacteria would not be overshadowed by the action of a flow of the water phase. The results of the experiments now reported show that to the contrary a distinct additive effect manifests itself, when both factors are allowed to act at the same time.

From the fact that the cultures used in these experiments were very old, and contained only a few active cells, we must conclude in agreement with ZoBell that a direct action of living cells on the solid phase or on the oil is inessential, and that the effect is primarily due to the physico-chemical changes brought about by the bacteria in the medium.

A further indication as to the nature of the responsible mechanism was found in the observation that in the experiments with the sterile medium the oil showed a stronger adhesion to the glass beads than in the other tests. In these a single rinsing in tap water left only traces of oil on the beads, while after an experiment with sterile medium, only petroleum ether could remove the adhering oil.

c. Changes in behaviour of the medium towards oil in consequence of bacterial growth.

In Part I it has already been shown that during growth in cultures of *Desulfovibrio desulfuricans* a distinct drop of surface tension occurs. There it has been proved that the analogous drop observed by ZoBell is not dependent on the presence of peptone in the medium, and will also take place in the synthetic medium used by us.

Next we have determined the interfacial tension of the media towards kerosene. As reported in Table III also this tension was found to be lowered significantly by the development of the bacteria. Qualitatively the same effect was observed by the addition of 0.1 % Tween 20 to the sterile medium.

But in other respects too bacterial growth led to relevant changes in the properties of the culture medium. A culture medium, about 1-2 weeks old, was found to be able to form stable emulsions, when 2 ml of it were shaken with 1 ml kerosene in a test tube. As suspensions of small particles, *e.g.* yeast cells (Bechhold *et al.* 1921), are known to do the same, the experiment was repeated with a filtrate obtained by filtration through a glass-sintered bacteria filter, and also with a suspension of washed cells collected by centrifugation of the culture. Both fluids showed emulsification after vigorous shaking. To the contrary no emulsion could be obtained with the sterile medium, so that a filtrable emulsifying agent must have been produced by the bacteria.

A drop of crude oil brought on a water surface spreads immediately. The same was observed for the surface of the sterile medium, but on a freshly poured out culture, several weeks old, initially no spreading took place. Fig. 3 gives an illustration of this difference in behaviour.

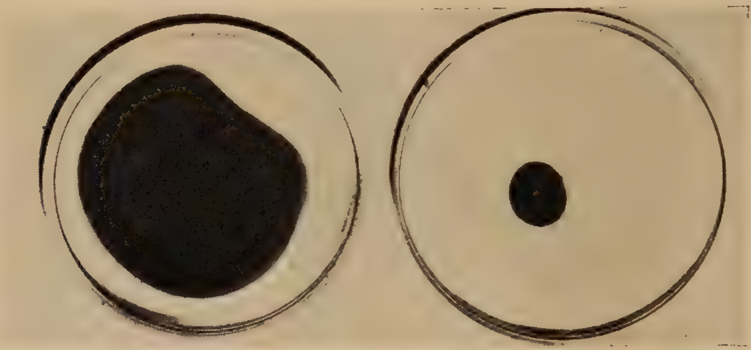

Fig. 3. Difference in spreading of a drop of oil on the surface of a) a sterile medium (left) and b) a 2 weeks old culture of *Desulfovibrio desulfuricans* in the same medium (right).

Only after 20-100 minutes a gradual spreading occurred showing that in this period interfacial relationships changed.

Furthermore differences in foaming properties were noted; the culture fluid produces on shaking a thin but distinct layer of foam, while the sterile medium does not produce any foam at all.

It must be stressed once more that all described phenomena have been observed in media to which no Ca was added. In cultures containing 0.1 % $CaCO_3$ or 0.1 % $CaCl_2$ surface tension measurement was hampered by the gradual formation of a film on the surface of the liquid. By moving the ring of the tensiometer out and into the fluid a clearly visible agglomerate was obtained on the surface. The film was formed within the time necessary for equilibration. Its formation was prevented by a preceding acidification with HCl; also in carbon dioxide atmosphere no film was formed. After acidification surface tension values proved to be the same as those in cultures without Ca. It was possible by pouring out the culture fluid into large flat dishes to harvest the film forming material by scraping the surface with strips of glass. Most of the substance was soluble in diluted HCl with gas formation. These observations can best be interpreted by accepting that $Ca(HCO_3)_2$, formed during growth, releases CO_2 on standing at the open air, thus forming an insoluble $CaCO_3$ layer. In addition it is likely that long chain fatty acids, if present, will contribute to the formation of the film as Ca salts.

The film in question shows interference colours, as oil films on water do. It is quite possible that its appearance is responsible for several equivocal reports on oil synthesis by bacteria.

Finally it seemed of interest to study the distribution of the cells between water and oil phase. Using the technique of MUDD and MUDD (1924) it was found that the cells never enter the oil phase. On a slide a drop of oil and a drop of a cell suspension were brought together by covering with a cover-glass. Observation in the dark field showed that after some hours accumulation of cells occurs in the oil-water interface; not all cells however were trapped, some highly motile cells were observed to leave the interface immediately after entering it. The accumulation of cells at the interface may well explain the observed emulsifying property of a cell suspension. It may be significant in oil release as surface active substances produced by the bacteria are bound to reach effective concentrations in the interface more rapidly than when no such accumulation occurs.

5. DISCUSSION.

ZOBELL did not publish any quantitative data on the amounts of oil released in his bottle experiments. BECK could not confirm ZOBELL's results with Bradford oil, as in his experiments no significant differences between sterile medium and grown cultures could be observed. The quantities of oil liberated in his experiments both in cultures and in controls amounted in no case to more than 100 mg from the 4—8 g, present in each bottle.

Our bottle experiments confirm the positive results of ZOBELL; in addition it has been shown by estimation of the amounts of oil released that the outcome of these experiments varies with the type of oil, the type of sand, and the oil content of the oil-sand mixture used.

The negative results obtained by BECK may well be due to a restricted growth in his experiments. Although the medium used by us was free from complex organic matter, it must be realized that its sulphate and lactate content make it especially suitable for extensive and prolonged growth of sulphate reducing bacteria. In all cases investigated the H_2S content was found to amount to approximately 1g/l after 3 weeks. BECK has used much poorer media and from the rather low final H_2S concentrations reported by him (< 400 mg/l), it can be concluded that growth in his cultures must have been rather restricted. It seems reasonable to accept that chemical changes in the medium due to bacterial development must have reached a certain degree, before oil release occurs.

As regards the mechanism of the bacterial oil release observed the following evidence has been established. Addition of a synthetic detergent to sterile medium makes this liquid behave qualitatively in the same way as when bacteria had been grown in it, both in bottle- and column experiments. Tween solutions as well as old bacterial cultures show not only a diminished surface tension, but also a distinct drop in interfacial tension towards kerosene. From the column experiments it can be concluded that action of living cells on oil or solid phase is not essential, and that physico-chemical changes brought about by the bacteria in their medium must be responsible. The weaker adhesion between oil and glass beads, when wetted by a culture medium and the observed emulsification, foaming and spreading phenomena, as recorded in section 4*c*, all indicate changes in capillary properties of the medium effected by the bacterial development. In view of these facts ZoBell's suggestion that bacterial activity in oil release may be due to changes in surface activity has become quite acceptable.

It must be emphasized that all experiments on oil release reported in the foregoing have been carried out under conditions that differ widely from those actually existing in oil reservoirs. Nevertheless it has been clearly shown, that there exist systems, consisting of an oil-containing solid phase combined with a water phase, in which oil can be released as a result of the development of sulphate reducing bacteria.

In reservoir engineering special laboratory techniques have been developed to test prospective injection liquids for secondary recovery on their performance in the reservoir. As it is very difficult to establish a laboratory equivalent of the situation in the reservoir, these tests do not permit definite predictions, but the indications obtained are important enough to be seriously considered before a decision is taken on the desirability of subsequent field tests. It would seem that there are no serious obstacles preventing the application of these test methods on bacterial cultures, since it has been shown that interaction of living cells on solid or liquid phases is not the essential active principle. The cultivation of the bacteria in the medium should be regarded as a method to obtain a solution of surface active matter, which solution ought to be as accessible for investigation on reservoir performance as the solutions of surface active agents that already have been studied. The results of our column experiments seem encouraging in so far as the biologically produced surface active matter was found to exert a positive influence, even under conditions in which a flowing aqueous phase was applied.

Moreover, it must not be lost out of sight that in the ability to produce surface active matter *in situ* bacteria possess a unique asset. It may well be that rather than the degree of surface activity of the injected liquid, the degree of penetration of this activity into the reservoir will determine the ultimate effect.

However, many more experiments, requiring highly specialised knowledge of reservoir engineering, will have to be done before predictions on the practical value of the bacterial method will be warranted.

Obviously there will be minimum demands for a medium that will still allow sufficient growth to bring about the desired effect.

It seems probable that the matter of costs will not be prohibitive as our results indicate that sewage along with sulphate might be a useful supplement to injection water. We also know that a cheap raw material as sulphite waste liquor is able to support growth of sulphate reducing bacteria (BANNINK and MULLER, 1952).

The question whether sulphate reducing bacteria are able to proliferate in oil reservoirs will probably present no difficulties as their presence has been demonstrated in many reservoirs.

It even seems likely that a special inoculation of the injection liquid will be unnecessary; the change in environmental conditions brought about by the injection may well lead to a natural accumulation of the organisms best adapted to these conditions, and amongst these undoubtedly sulphate reducing bacteria will prevail.

There are, however, two more or less essential factors which may prove to be fatal for a practical utilization. Actually the first relations between bacteriology and oil recovery were far from pleasant, and consisted in the plugging of the reservoirs caused by bacterial growth in injected water; for this reason even addition of bactericides is no uncommon practice. However, in our case plugging by cell material may not present a serious danger in view of the rapid autolysis encountered in some strains of sulphate reducing bacteria.

Secondly porosity and permeability may be lowered by precipitates formed by additives to the injected water, or by metabolic products of the bacteria. Addition of sulphate might give rise to precipitation of $CaSO_4$ or $BaSO_4$, whilst the H_2S produced may lead to the formation of FeS. For this reason careful study for each reservoir will have to decide, whether a composition for an injection fluid can be found which is compatible with both the demands of the bacteria and those made by the chemical composition of the reservoir rock and that of the water.

Whether the migration of oil from its original source bed to the present-day accumulations, has been influenced by bacteria still forms an unanswered question. If a sufficient growth of sulphate reducing bacteria has taken place in a water phase in contact with oil in a porous material, it seems very likely that these bacteria will have aided in oil migration, although an evaluation of the importance of this factor will remain difficult.

Summary.

In general agreement with ZoBell's results it has been found that cultures of *Desulfovibrio desulfuricans* are able to release both Trinidad and Schoonebeek crude oils from oil-sand mixtures at atmospheric pressure and 30° C. The amount of oil liberated after 3 weeks in stationary bottle cultures, in excess of the quantities released in control experiments, varied from 5—35 % of the oil originally present in the oil-sand mixture.

Under identical conditions oil release was obtained with a sterile medium to which Tween 20 had been added.

In the synthetic medium used, growth of the bacteria resulted as well in a markedly lowered surface tension, as in a lowered interfacial tension towards kerosene.

Experiments on oil release from a glass bead column by a slow current of an aqueous phase have been carried out. On comparing: *a.* sterile medium, *b.* medium in which sulphate reducing bacteria had been grown, *c.* sterile medium to which Tween 20 had been added, it was found that, in agreement with the result of the bottle experiments, the two latter liquids showed distinctly superior oil releasing powers.

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(National Institute of Public Health, Utrecht, Netherlands).

A ROLL-TUBE TECHNIC FOR THE STUDY BY FLUORESCENCE-MICROSCOPY OF THE GROWTH OF MYCOBACTERIA

by

B. HYLKEMA and **J. H. BEKKER** [1])

(Received March 15, 1954).

The roll-tube technic as described below resulted from a study of the reliability of the slide culture method of PRYCE (1941) for the rapid bacteriological diagnosis of tuberculosis. According to this method a film of sputum or other pathological material is dried on a glass slide, incubated in contact with hemolysed blood and examined for micro-colonies of tubercle bacteria. As some difficulties are attached to this method (e.g. the fixation of the smears on the slides) a technic was developed by which greater amounts of material could be investigated under controlled conditions favourable for the growth of tubercle bacteria. This technic is a special modification of the roll-tube method originally described by VON ESMARCK (1886) and KÖRBER (1894) and improved by GEE (1932), THOMPSON (1934), JULIUS (1938), MOIR and THOMSON (1950) and others.

Roll-tube apparatus.

The apparatus for preparing the roll-tube media consists of a series of fibre rolls placed parallelly and horizontally into a frame. Driven by an electric motor, the rolls turn in the same direction around axis fixed in accurately adjusted ball-bearings (fig. 1).

The culture tubes, containing a certain amount of medium and plugged with sterile cotton, are placed between the rolls. The tubes revolve between the rolls and are kept in their place by a slight inclination given to the rolls and by the funnel-shaped mouth of the tube, while their weight prevents a move on the roll-floor. The apparatus is hinged on to the base and before placing the tubes on the rolls the inclination of the rolls is slightly increased.

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[1]) Presented at the meeting of the Netherlands Society for Microbiology of November 27, 1953 at Amsterdam.

After some second's turning the medium is deposited against the wall of the tube and the apparatus is brought back into the original position. If the tubes are handled with sufficient care this precaution against a contact of the medium with the cotton plug may probably be dispensed with. The solidification of the medium is accelerated by the cool air current caused by the turning rolls.

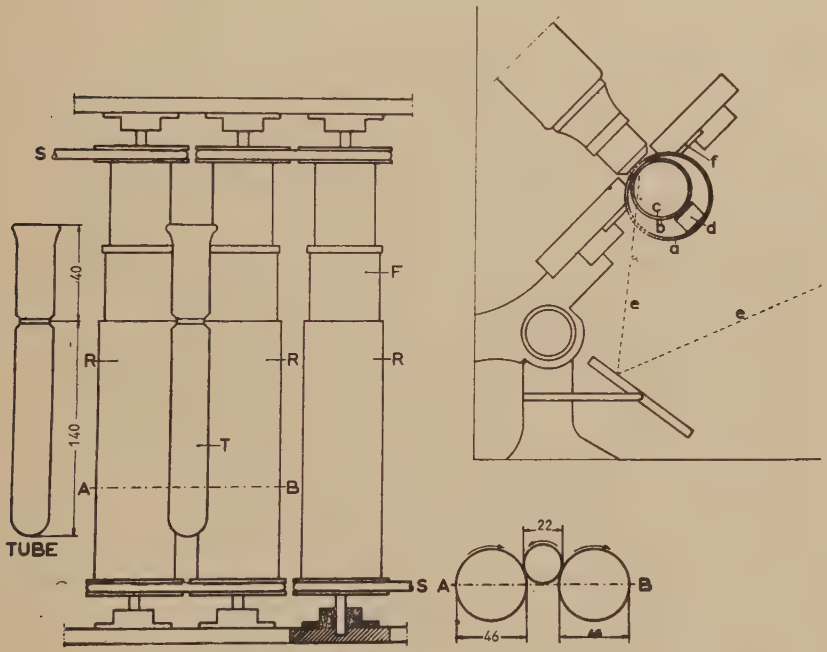

Fig. 1. Design and cross-sections of the roll-tube apparatus, and of the reading microscope. The culture tubes (T) are placed between the rollers (R), which are connected with chords (S) used for the transmission of motion. F: excavation made to save grease pencil inscriptions on revolving tubes. Reading microscope: The culture tube (b) in the holder (a) is pressed against the observation window by a spring (d). c: inner surface agar, e: direction of the bundle of light, f: carriage.

Culture tube.

The culture tubes measure 180 × 22 mm and a constriction of some millimeters is made at 40 mm from the funnel-shaped mouth.

The advantage of this roll-tube apparatus is in that the tubes need not be standardized, the normal variation in length and diameter having no influence; even the constrictions need not be uniform. It is obvious that the axis of the upper part and the body of the tube must be in a direct line.

Normal culture tubes can simply be transformed into roll-tubes by making a constriction and by dilatation of the upper edge.

Culture medium.

For use in the roll-tube technic the medium has to be clear and transparent, and for solidifying against the wall of the tube the medium must contain a certain amount of agar. By distributing 10 ml of melted medium in the tubes after solidification a medium with a thickness of 1.3 mm and an area of 70 cm^2 is obtained. A basal medium of 10% beef serum agar is used, the exact composition depends on the nature of the experiment [1] [2]. After inoculation the tubes are rolled again in order to distribute the inoculum evenly on the surface of the medium.

Reading microscope.

The principle of our roll-tube technic is the microscopical examination of the colonies of mycobacteria which fluoresce against a dark background as a result of a special illumination. For this purpose the tube is placed in a black metal holder with two long rectangular windows, one for the examination of the colonies, the other for the entrance of the light. The holder can be placed under the stage of a microscope and be pulled over a tubular lamp beside the microscope for the macroscopical examination of the colonies. After orientation on the millimeter scale on the holder the suspected colony is placed in the field of the microscope for further examination (fig. 2).

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[1] The basal medium contains per 1000 ml: 7.5 g bisodium phosphate 12 aq, 0.55 g monopotassium phosphate, 0.25 g magnesium sulphate 7 aq, 3 g sodium citrate, 1 g asparagin, 1 g Bacto-casitone, 5 mg ferric ammonium citrate, 0.5 mg calcium chloride, 0.1 mg zinc sulphate, 0.1 mg copper sulphate, 3 mg malachite green, 6 ml glycerol (bidistilled), 16 g Bacto-agar (washed in distilled water) and 100 ml inactivated beef serum (kept a month or longer at 3°C. and filtrated by Seitz E.K.); pH of the medium 7.4—7.5.

[2] An investigation into the nutritional requirements for the growth of tubercle bacteria in roll-tubes will be published shortly.

As source of illumination a Leitz-Monla-lamp is used, the light passing through a blue filter consisting of a cuvette of 1 cm diameter containing a 0.5% ammoniacal copper sulphate solution.

This bundle of blue light is thrown through the window in the holder into the culture tube and is passing through the colonies at an angle of incidence of 33° (fig. 1). The fluorescence phenomena are studied through the microscope (objective 10 ×, ocular 5 ×).

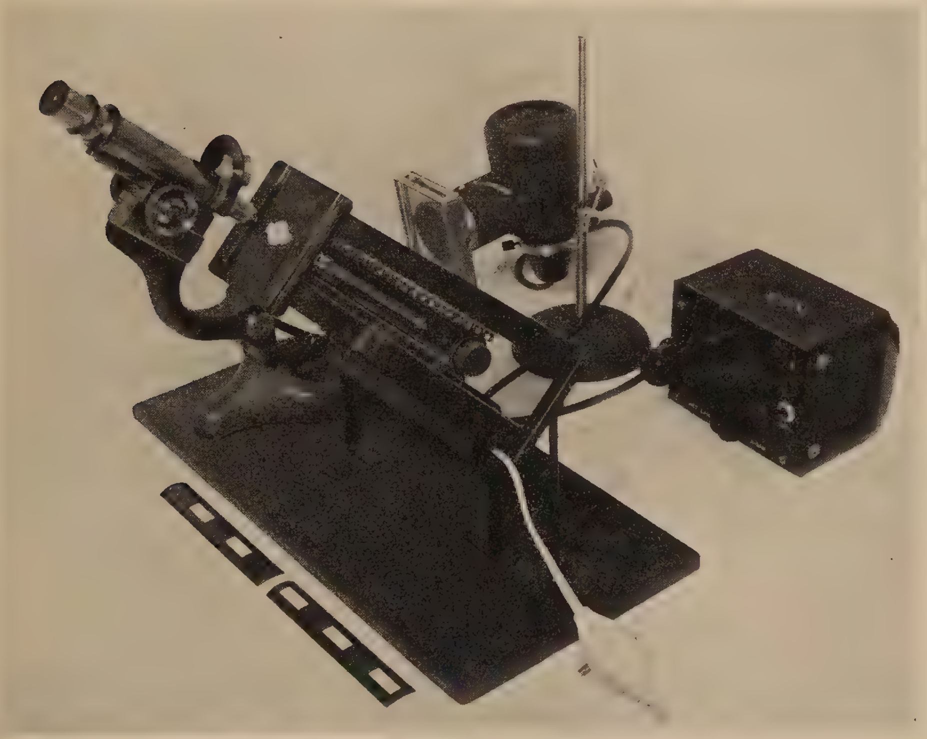

Fig. 2. Reading microscope. The holder containing a culture tube is placed on a carriage with millimeter scale and can be pushed under the stage of the microscope. For counting the number of the colonies the observation window is closed by the shutters with apertures seen before the base of the microscope.

Colonies of acid fast bacteria fluoresce with a golden-yellow colour; colonies of other micro-organisms have a blue colour, except those of yeast and fungi, which also fluoresce with a yellow colour, but which can easily be distinguished from those of mycobacteria. Very thin structures of micro-colonies of mycobacteria are colourless (not blue) with yellow fluorescing stripes and bars where the light passes thicker bundles of bacteria. The optimal illumination gives contrastful pictures of the structure of the colonies, whereby the colonies of tubercle bacteria can easily be recognized.

Conclusion.

The described roll-tube technic has some advantages above other methods for the study of microcultures of mycobacteria [ØRSKOV and ENGBAEK (1951), ENGBAEK (1952), CHRISTENSEN *et al.* (1953)].

1. By a special method of illumination use is made of the fluorescence of mycobacteria, by which their colonies can be differentiated from the colonies of other micro-organisms; futhermore a differentiation is possible between the colonies of tubercle bacteria and those of acid fast saprophytes.
2. The clear and transparent culture medium fixed on to the wall of the culture tube has a large area so that it can be used in those cases where plate culture methods are usually preferred.
3. The estimation of the number and the size of the colonies can easily be accomplished.
4. By varying the composition of the medium the influence of growth stimulating and growth inhibiting factors can be investigated by studying the development of the micro-cultures of mycobacteria.

Summary.

A description is given of a roll-tube technic for the study of the growth of mycobacteria by means of fluorescence-microscopy.

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AN APPARATUS FOR THE CONTINUOUS CULTURE OF BACTERIA AT CONSTANT GENERATION TIMES

by

P. G. DE HAAN and **K. C. WINKLER**

(Received May 19, 1954).

Several devices to culture bacteria continuously at constant generation times have been described (JORDAN and JACOBS, 1944; NOVICK and SZILARD, 1950; MONOD, 1950; BRYSON *et al.*, 1949, 1951). Our trials date from 1948 and have since largely been influenced by the work of NOVICK and SZILARD. Like others we have been hampered however by the lack of detailed information and the scanty instructions for use. We hope with this report which does not claim any priority or originality, to serve with our experience those who wish to make use of this important and interesting tool. The principle of "continuous culture" is of course to increase the volume of the medium at the same rate as bacterial reproduction i.e. to double the volume in one generation time. To circumvent inordinately large volumes only a small sample is kept after each dilution. A practical solution is to drop fresh medium (at a constant rate) into a culture vessel with an overflow so that the amount of fluid in the vessel is held constant.

DESCRIPTION OF THE APPARATUS AND INSTRUCTIONS FOR USE.

The apparatus is shown diagrammatically in fig. 1 (compare also fig. 2). A is the culture vessel into which a constant inflow of fresh medium is entered at *i* (external diameter about 4 mm, weight of one droplet about 70 mg). Through the overflow O an equal amount of fluid per unit time is discarded. The fluid in the vessel is stirred by a current of sterile air through the tube D. Vessel B contains the supply of fresh medium, C is the pressure stabilizer. Compressed air is introduced at E. The pressure is stabilized in

C at a value dependent on the height of the watercolumn h_1 in C. The air then passes the capillary throttle F and the cotton filter G and enters the container B at P in tiny bubbles. As the container B is closed, the rate of flow into the culture vessel A is independent of the amount of fluid in B and is only determined by $h_1 - h_2$; the rate of flow can thus be regulated by changing the value of h_1.

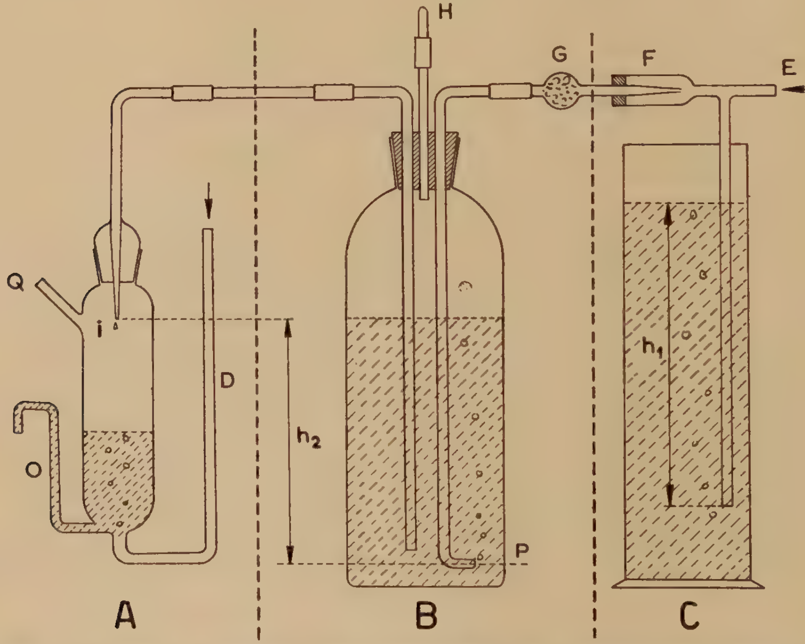

Fig. 1. Diagram, showing the apparatus for the continuous culture of bacteria. A is the culture vessel; B the container with liquid medium and C the pressure stabilizer. Compressed air enters at E. The value $h_1 - h_2$ determines the flow rate of the medium at i. As h_2 is constant, the flow rate can be regulated by changing the value of h_1.

To guarantee the constancy of h_1 and h_2 especially at low flow rates some precautions are necessary.

1. The water in C evaporates during long term experiments, care should be taken to keep the level in C constant.
2. The bubbles of air escaping in C produce minor pressure fluctuations which imperil the constancy of the flow of tiny bubbles at P. The capillary F serves as a throttle to stabilize the flow rate.
3. After the escape of a bubble from P the level of the fluid in this tube rises several millimeters thereby reducing h_2. To stabilize h_2

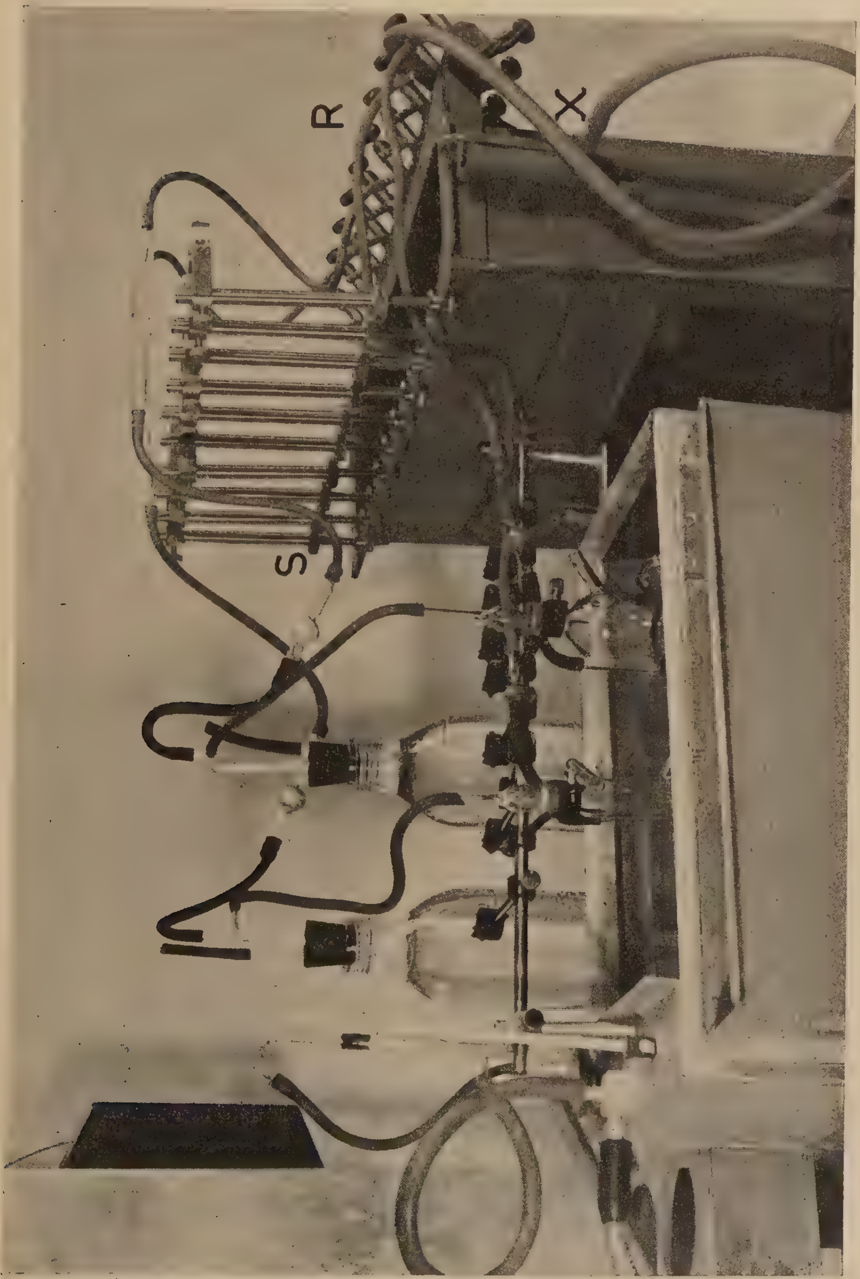

Fig. 2. The continuous culture apparatus in use.

The apparatus is built for the simultaneous use of more than one culture vessel. Two culture vessels are shown, each with its own container. Pressure air from the main enters the system at the right (not visible) and is approximately reduced to the required pressure by one of the reducing valves R. These valves are also used to control the flow rate of the aeration of the culture vessel. Finer pressure regulation is obtained by varying h_1 by means of the screws at the left side of the stabilizer (S). The level of the water in the stabilizer is kept constant in the usual way (X).

the tube P should be fitted with a horizontal arm or with a horizontal spiral ending in a fine point.

4. The air outlet Q should not be closed with a cotton plug which easily takes up moisture. The increased resistance of the damp plug results in an increased pressure in A so that part of the contents of the vessel or even the whole volume of fluid will be pressed through the overflow.
   In working with pathogens the air current through Q should be considered contaminated. It can easily be sterilized by passing it through a small chimney pipe in which a suitable ultra violet light source in installed.
5. The apparatus should be kept at constant temperature as the pressure in B fluctuates with temperature. We keep the vessel A in a waterbath and the whole apparatus in an incubator room.

Taking these precautions into account it is possible to obtain constant flow rates between one drop in five or one drop in thirty seconds. To obtain a slower rate of dilution a larger vessel should be used (see table 1).

TABLE 1.

The constants of a number of suitable culture vessels.

Vessel	Vessel constant $\frac{1}{x}\lg.\frac{V_0}{V_0 + x}$	Volume approximately	Generation time at a flow rate of one droplet in 25″
2	—0.005558	5 ml	22.5 minutes
4	—0.002335	13 ml	53 minutes
7	—0.000844	36 ml	149 minutes

The culture vessel A, the container B and the rubber stopper of B with its connecting tubes are sterilized separately. After sterilization B is filled with sterile medium and closed with the rubber stopper. During closing the plug H should be removed to prevent compression of the air in B (and the risk that the rise of the fluid in the connecting tubes would wet the cotton in G). The vessel A is either filled with a pre-grown culture and assembled or after assembling the culture vessel is filled from B and inoculated through Q. The air current through A is then started and after a suitable time the air current E is switched on. Samples are taken from the overflow O.

THEORETICAL CONSIDERATIONS.

Though it is possible to secure a continual dropwise inflow of medium into the vessel A the outflow is more discontinuous and happens in portions of several droplets. Depending on the diameter of the vessel between 3 to 10 droplets have to be added before the level in the vessel A rises sufficiently to overcome the surface tension at O. At that time a corresponding volume leaves the vessel. This phenomenon should be taken into consideration in all calculations concerning this kind of apparatus.

Suppose a flow rate of one droplet in t seconds and a culture vessel with a volume of V_0 droplets. Suppose that x droplets have to be added in xt seconds before the overflow starts. At the time xt the volume has increased to $(V_0 + x)$ and drops again to V_0. $\frac{V_0}{V_0 + x}$ is the dilution factor (p) in one cycle during the time xt.

The number of bacteria (N_0) in the vessel increases during this time to $N'_{xt} = N_0 e^{kxt}$. After the overflow the number is reduced to

$$N_{xt} = \frac{V_0}{V_0 + x} N'_{xt} = \frac{V_0}{V_0 + x} N_0 e^{kxt} = p N_0 e^{kxt} \tag{1}$$

This cycle is repeated so that after T seconds or T/xt cycles the number of bacteria is

$$N_T = N_0 p^{\frac{T}{xt}} \times e^{kT} \tag{2}$$

or writing 10^K for e^k:

$$N_T = N_0 \, p^{\frac{T}{xt}} \times 10^{KT}$$

$$\lg N_T = \lg N_0 + \frac{T}{xt} \lg p + KT.$$

To obtain a steady state with a constant number of bacteria ($N_T = N_0$), $\frac{1}{xt} \lg p$ should be equal to K or the increase in the number of bacteria should be equalled by the dilution. We will call $\frac{1}{x} \lg p$ the vessel constant. With known vessel constant it is theoretically possible to regulate the flow rate for each value of K.

If a not too small number of bacteria is inoculated logarithmic growth will start after a certain lag. With a high flow rate $\frac{1}{xt} \lg p$

will be greater than K and the number of bacteria will decrease, the bacteria will be diluted out. In the reverse case the number of bacteria will increase.

A regulation of the flow rate for the value of K during logarithmic growth is cumbersome and rarely possible. At the end of the logarithmic growth the growth rate slows down however and K decreases. On starting the flow rate in the vessel A at a certain value so that $\frac{1}{xt} \lg p < K$ the number of bacteria will increase until K decreases. As soon as a value $K' = \frac{1}{xt} \lg p$ is reached a steady state is established and the number of bacteria remains constant. In the steady state the bacteria reproduce at a velocity corresponding to flow rate and vessel constant, that is with a definite generation time (G.T.)

$$\text{G.T.} = \frac{0.30103}{K'} = \frac{0.30103 \times xt}{\lg p}$$

Thus, with known vessel constant it is easily possible to adjust the flow rate for any generation time (above that during fast logarithmic growth). High flow rates correspond with short generation times and low levels of the number of bacteria in the vessel. Low flow rates correspond with long generation times and a high level of the number of bacteria.

DETERMINATION OF THE VESSEL CONSTANT.

The vessel constant is determined by the volume V_0 and by the number of inflowing droplets x, necessary to start the overflow. The value of x can be estimated experimentally by taking the mean of a number of observations. It is more difficult to estimate V_0. It is of course possible to measure the volume of the vessel (without the side arm) in the apparatus at rest. During use however the aeration current reduces the volume of fluid that is actually present. It is possible to determine this actual volume by several methods. The best method is to obtain the vessel constant $\frac{1}{x} \lg p$ directly.

The vessel A is filled with 0.1 n hydrochlorid acid to which a suitable indicator had been added (phenolphthalein).

As soon as the aeration current through A is started a certain amount of fluid overflows and the actual volume V_0 is present. Sodium hydroxide (0.1 n) is now added from the supply flask B at a constant flow rate (one droplet in t seconds) and the time T necessary for neutralisation is noted. The vessel constant can now be calculated as follows:

Suppose a and b to be the strength of the acid and sodium-hydroxide respectively and let us write p for the dilution factor $\frac{V_0}{V_0 + x}$. Then after the addition of x droplets of sodium hydroxide the amount of free acid in the vessel A will be $aV_0 - bx$ and after overflow

$$p\,(aV_0 - bx) = paV_0 - pbx$$

after the second cycle we have

$$(paV_0 - pbx - bx)\,p = p^2aV_0 - p^2bx - pbx$$

and after the nth cycle

$$p^naV_0 - bx\,(p^n + p^{n+1} \ldots . + p)$$

or taking the sum of the geometric series of p

$$p^naV_0 - bx \times p\,\frac{1 - p^n}{1 - p}$$

Suppose that neutralisation occurs after the nth cycle the amount of acid will then be zero or

$$p^naV_0 = bx \times p\,\frac{1 - p^n}{1 - p}$$

In writing again $\frac{V_0}{V_0 + x}$ for p some simple transformations give

$$\frac{b}{a + b} = \left(\frac{V_0}{V_0 + x}\right)^n$$

at neutralisation n will be $\frac{T}{xt}$ so that the vessel constant will be

$$\frac{1}{x}\lg\frac{V_0}{V_0 + x} = \frac{t}{T}\lg\frac{b}{a + b}$$

An example of suitable culture vessels with volume and constants is given in table 1.

GROWTH IN MEDIA WITHOUT SPECIAL LIMITING FACTORS.

In fig. 3 an example is given of the growth of *E. coli* B in liquid minimal medium. The flow rate was adjusted for steady state with a generation time of 90 minutes. After inoculation, during the lag of about one hour the inoculated bacteria are diluted out.

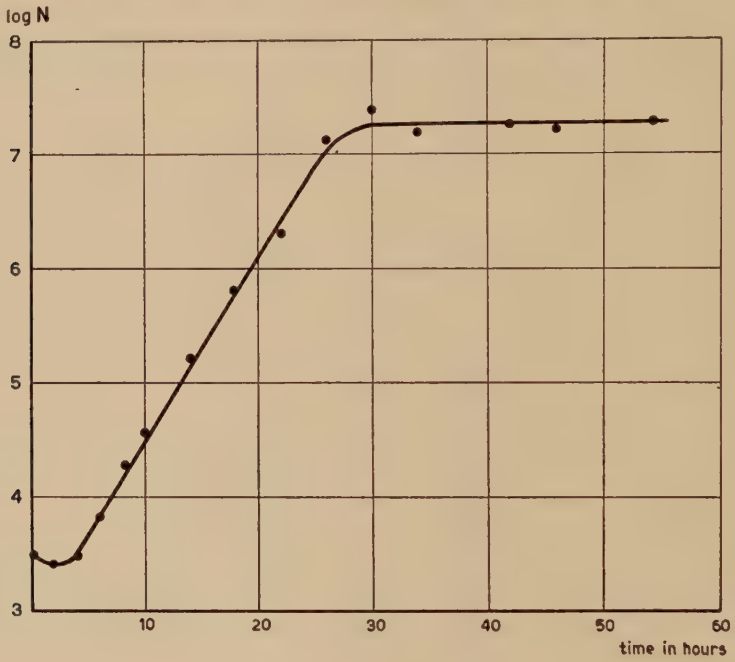

Fig. 3. The growth of *E. coli* strain B in minimal medium in the continuous culture apparatus. The number of bacteria per standard droplet of 1/30 g is counted in the overflowing liquid. During the logarithmic phase the bacteria grow with a generation time of 47 minutes. After 30 hours a steady state is reached (generation time 90 minutes).

During exponential growth the bacteria are dividing with a generation time of 47 minutes. The generation time obtained from the data in fig. 3 should of course be corrected for the dilution factor. The number of generations $n = \frac{\lg N_2 - \lg N_1}{\lg 2} + \frac{T_2 - T_1}{90}$. At a concentration of about 10^7 bacteria per standard droplet the growth rate decreases until a steady state with a constant number of bacteria is reached.

Theoretically there exist two possible mechanisms by which the number of bacteria is kept constant. Either all bacteria divide at a generation time of ninety minutes or a constant fraction of the population divides at a higher rate while the other fraction though viable in the plating medium does not multiply in the vessel. We will show later that the former possibility is nearer the truth.

GROWTH IN MEDIA WITH SPECIAL LIMITING FACTORS.

Bacteria growing in the apparatus will develop logarithmically until K decreases to equal the dilution factor. The decrease of K will be caused just as in a normal culture by a complex of factors (pH, decrease of food supply, accumulation of metabolic products etc.). It is possible however to use media in which the supply of one known substance is the limiting factor. This method greatly increases the possibilities of the apparatus for continuous growth (NOVICK and SZILARD, 1950; MONOD, 1950).

To realize what happens it is useful to discuss the case of a normal

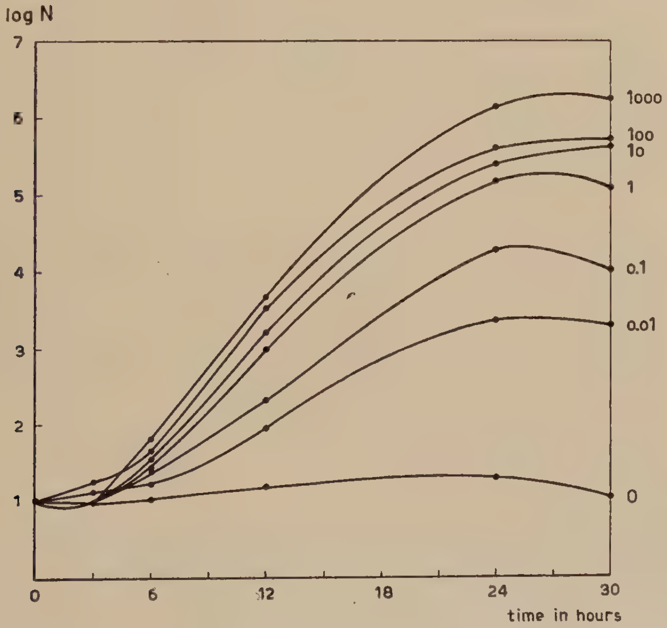

Fig. 4. The growth of a methionine dependent mutant of *E. coli* B on minimal medium with various concentrations of methionine. The number at each curve indicates the amount of methionine in μg per 10 ml of medium. Log N gives the number of viable bacteria per standard droplet of 0.03 g.

culture first. The growth velocity of for instance a methionine dependent mutant will be a function of the methionine concentration $K = f(c)$. In the presence of an excess of methionine the growth rate will be high and after a short logarithmic growth the stationary phase will be reached. The number of bacteria at this level will be independent of the original methionine concentration. With smaller initial concentrations the bacteria start growing at a lower rate (fig. 4). This rate is constant until the amount of methionine decreases perceptibly and the bacteria enter the stationary phase at a lower level because the residual amount of methionine does not permit further reproduction. In this case the metabolic apparatus of the bacteria is not harmed by toxic products and unchanged by adaptive processes but further reproduction is exclusively limited by the lack of methionine.

In the continuous culture analogous phenomena occur. Supplying a medium with a limited amount of the special growth factor, at such a rate that $f(c) > \frac{1}{xt} \lg p$ the number of bacteria will increase. The increasing number of bacteria will use increasing amounts of the limiting factor until the actual concentration drops notwithstanding the continual supply, so as to limit growth and reduce K. In this way a steady state will be obtained as soon as

$$\frac{1}{xt} \lg p = f(c).$$

The generation time in this steady state will again be dependent on the flow rate. The level of the number of viable bacteria (N_{max}) still depends on the flow rate but is now also controlled by the initial concentration (C_0) of the growth limiting substance in the supply vessel, low values of C_0 corresponding with low values of N_{max}.

RESULTS.

The apparatus was used to study the propagation of bacteriophage in bacteria growing at varying generation times. These results will be discussed in a separate paper.

POSSIBILITIES OF THE CONTINUOUS CULTURE.

A. Pure cultures.

1. The pure culture of the bacteriologist is an artifact. In nature

food supply will often be discontinuous in space and time (STANIER, 1952) so that the bacteria show spasms of growth analogous to the classical "growth curve". Just as often however the food supply will be more or less continuous at a low level over reasonable times. This means that bacteria in their natural habitat often grow with generation times intermediate between the abnormal short ones of "logarithmic growth" and the abnormal long ones in the half starved condition we call the "stationary phase". It is well known that the properties and the metabolism of most bacteria change at the end of the "logarithmic" phase. The continuous culture permits the study of bacteria at these intermediate generation times which occur only for very short periods in classical pure cultures.

2. For the study of all kind of population phenomena a prolongued growth at constant rate is of primary importance. The apparatus can for instance be used (or has been used) for:

a. the isolation of resistant mutants (BRYSON, 1952);

b. the study of mutation frequency in relation to generation time and food supply (NOVICK and SZILARD, 1951);

c. for studies of genetic equilibria and population shifts (periodic selection) which up till now required frequent subcultures (cf. STOCKER, 1949).

B. Mixed cultures.

The interrelationships of two or more bacterial species in one medium can be studied under conditions much nearer to those in a natural flora, thereby carrying bacteriology nearer to the very complex but interesting level marked by STEPHENSON as the final goal of bacteriology.

Acknowledgements.

We are greatly indebted to Mr A. KERSSEN for the technical construction of the apparatus and to Miss H. S. FELIX for efficient practical assistance during the experiments.

Summary.

An apparatus for continuous culture of bacteria at constant generation times is described, analogous to NOVICK and SZILARD'S

"chemostat" and the apparatus of MONOD. Detailed instructions for the use of the apparatus are given and a method for the determination of the vessel constant is described. The theory and the interesting possibilities of the apparatus for a number of fundamental problems in bacteriology are shortly discussed.

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DIFFERENTIATION OF *CORYNEBACTERIUM DIPHTHERIAE* OF THE *MITIS* TYPE FOUND IN DIPHTHERIA AND OZAENA

II. THE RATE OF RAPIDITY OF GLUCOSE FERMENTATION BY *C. DIPHTHERIAE* TYPE *MITIS* AND *C. BELFANTI*

by

VLADIMIR BEZJAK

(Received May 24, 1954).

One of the methods recently used by some authors in typing the strains of *C. diphtheriae* was that described by Parsons and Frobisher (1951). With their technique the rate of rapidity of glucose fermentation is estimated and the *intermedius* type of *C. diphtheriae* could easily be differentiated from other types of diphtheria organisms by its slow fermentation of glucose in heart-infusion glucose broth.

Freeman and Minzel (1952) proposed a new scheme for the classification of *C. diphtheriae* strains based on the method given by Parsons and Frobisher. The types *gravis* and *mitis* are completely changing the colour of the indicator in 24 to 72 hours, while *intermedius* strains, when freshly isolated, will ferment glucose more slowly and not before 8 to 10 days have elapsed.

In connection with my previous report, i.e. on the existence of a *Corynebacterium* which could be differentiated from an atoxic *C. diphtheriae* type *mitis* only by its failure to reduce nitrates (the so-called *C. belfanti*), it seemed necessary and interesting to ascertain whether there would exist a difference between these two Corynebacteria when using the method of Parsons and Frobisher.

MATERIALS AND METHODS.

Eighty strains of *C. belfanti*, i.e. atoxic hemolytic strains of a *Corynebacterium* which fermented only glucose, but did not reduce nitrates, freshly isolated from ozaena patients, were tested for the rate of rapidity of glucose fermentation according to the method given by PARSONS and FROBISHER.

The essential part of the technique described by these authors is the avoidance of any serum in the medium, otherwise false positive reactions may result.

Therefore, prior to testing, the strains were grown for 48 hours in Difco heart-infusion broth with brom-cresol-purple as indicator (pH 7.8). Using a capillary pipette 1—2 drops of this culture then were inoculated into the same medium provided with 1% glucose and the tubes were incubated at 37° C. for 10 days. The tubes were daily observed for the change of the colour of the indicator. Only tubes which would show a complete change of colour were designed as positive.

The medium was kept in Wassermann tubes in volumes of 3 ml. To each tube 0.3 ml of a 10% sterile aqueous solution of chemically pure glucose was added in order to constitute a 1% glucose medium for testing.

For comparison 80 strains of *C. diphtheriae* type *mitis*, all freshly isolated from diphtheria cases or carriers, were also tested for the rate of rapidity of glucose fermentation, using the same technique.

RESULTS.

The results of the rate of rapidity of glucose fermentation test, carried out with 80 strains of *C. diphtheriae* type *mitis* as well as 80 strains of the so-called *C. belfanti* are shown in Table 1.

TABLE 1.

The results of testing the rapidity of glucose fermentation.

strains	% glucose fermented in:							
	1 day	2 days	3 days	4 days	5 days	6 days	8 days	9 days
C. diphtheriae type *mitis*	3/3.8	69/86.2	8/10	—	—	—	—	—
C. belfanti	5/6.2	6/7.5	21/26.2	29/36.2	7/8.7	8/10	2/2.5	3/3.8

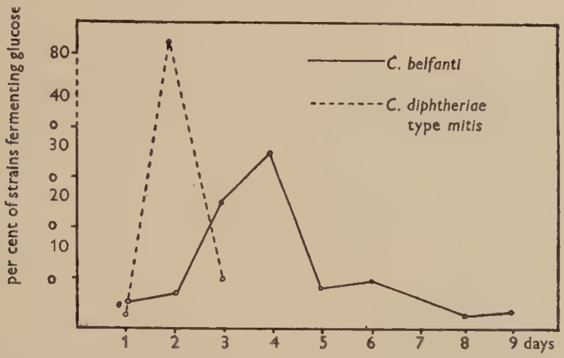

Fig. 1. The rate of rapidity of glucose fermentation by *C. diphtheriae* type *mitis* and *C. belfanti* strains.

All *mitis* strains appear to ferment glucose in the period of 3 days, while the greater majority of strains (90%) fermented glucose in two days already.

When compared with *C. diphtheriae* type *mitis* the strains of *C. belfanti* fermented glucose more slowly, some of them needing even a period of 9 days. The majority of *C. belfanti* strains fermented glucose in 3 to 4 days (62.4 per cent), whereas after 2 days only 13.7 per cent of strains gave a positive reaction.

All the results are also presented in Fig. 1, where percentages of strains fermenting glucose were plotted against the time in days.

DISCUSSION.

The rate of rapidity of glucose fermentation test was successfully introduced by PARSONS and FROBISHER for the differentiation of the *minimus* i.e. *intermedius* type of *C. diphtheriae*, which showed positive reaction in 8 to 10 days in contrast to *gravis* and *mitis* strains which usually gave positive results in 1 or 2 days.

In our experiments on the rate of rapidity of glucose fermentation *C. belfanti* showed marked difference from *C. diphtheriae* type *mitis*. Therefore, atoxic strains of *C. diphtheriae* type *mitis* could be differentiated into two groups: one which reduces nitrates and ferments glucose in 2 or 3 days (using the method of PARSONS and FROBISHER) and the other which does not reduce nitrates and needs 3 or more days for the fermentation of glucose. The latter group is represented by strains which we design throughout this study as *C. belfanti*.

Summary.

Eighty strains of *C. diphtheriae* type *mitis* and 80 *C. belfanti* strains were tested for the rate of rapidity of glucose fermentation according to the method of PARSONS and FROBISHER.

90% of *C. diphtheriae mitis* strains, in contrast to only 13.7% of *C. belfanti* strains, fermented glucose in 1 to 2 days. 76% of *C. belfanti* strains fermented glucose in 3 to 4 days, whereas some strains needed 8 to 9 days to complete the fermentation.

So the results of this test revealed next to that of nitrate reduction, a further difference between the strains of *C. diphtheriae* type *mitis* found in diphtheria and ozaena.

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CAULOBACTER

ITS MORPHOGENESIS, TAXONOMY AND PARASITISM

by

A. L. HOUWINK

(Received July 3, 1954).

Caulobacter is the only genus described in the family *Caulobacteriaceae*. The names of both the genus and the family were given by Henrici and Johnson (1935). Out of several types of bacteria belonging to the genus, only one was described as *Caulobacter vibrioides*.

The definition of the *Caulobacteriaceae* is:

> "Stalked bacteria, the long axis of the elongated cells coinciding with the axis of the stalk. Stalks are slender, flagellum-like, often attached to the substrate by a button-like holdfast, unbranched. Multiplication of cells by transverse-binary fission. The outermost cell of a pair may form a stalk before cell division is complete. Periphytic, growing upon submerged surfaces".

The *Caulobacteriaceae* were classed in the order *Caulobacteriales* Henrici et Johnson, which also included *Nevskiaceae*, *Gallionellaceae* and *Pasteuriaceae*. The order was defined as follows:

> "Bacteria growing characteristically upon stalks. The cells are asymmetrical in that gum, ferric hydroxide, or other material, is secreted from one side or from one end to form the stalk. Multiplying typically by transverse binary fission; in one family by budding and longitudinal fission. In some species stalks may be very short or absent, the cells connected directly to the substrate or to each other by holdfasts. Cells occur singly or in pairs, never in chains or filaments; not ensheathed. Typically aquatic in habitat; some may be parasitic in animals".

Bergey's Manual Vth ed. followed Henrici and Johnson's classification. In the VIth ed., however, the *Caulobacteriaceae*, *Nevskiaceae* and *Gallionellaceae* were classed among the *Eubacteriales* as a new suborder *Caulobacteriineae* Breed, Murray et Hitchens. In addition to the families already mentioned, the suborder included the *Siderocapsaceae* Pribram. The *Pasteuriaceae*, by reason of being considered fundamentally different from the other families, were placed in an appendix.

The name of the suborder *Caulobacteriineae* suggests that all the species included have stalks, as had most of the *Caulobacteriales*. Stalks are lacking, however, in the newly included family *Siderocapsaceae*. The definition of the suborder is therefore not identical with that of the *Caulobacteriales* but runs as follows:

> „Non-filamentous, attached bacteria growing characteristically upon stalks, sometimes sessile. The stalked cells are asymmetrical in that gum, ferric hydroxide or other material is secreted from one side or one end of the cell to form the stalk. Multiply by transverse fission. In some species the stalks are very short or absent. In the latter case the cells may be attached directly to the substrate in a zoogloeic mass. Cells occur singly, in pairs or short chains, never in filaments; not ensheathed. Non-sporeforming. Typically aquatic in habitat".

As a gradual transition from the sessile into the stalked habit is well conceivable, one can agree with the editors of Bergey's Manual that the emphasis placed upon the presence of a stalk by HENRICI and JOHNSON seems artificial.

A grave objection, however, should be raised against one sentence common to the definitions of *Caulobacteriales* and *Caulobacteriineae*: "The (stalked) cells are asymmetrical in that gum, ferric hydroxide or other material is secreted from one side or one end of the cell to form the stalk". This is rather misleading. A stalk need not be made of some secreted material. Equally well it might be part of the bacterial cell. As has already been remarked by BISSET (1952), the difference between a bacterium in which the stalk may be considered a true organ, and one with a stalk made of gum or ferric hydroxide would offer a better motive for classing them each apart, than the attached habit they have in common could be a reason for linking them up in an order or suborder.

Caulobacter may be found in fresh water, *e.g.*, in tapwater, and can be isolated on peptone agar plates, if the inoculating material contains a fairly high percentage of the organisms. Young colonies can be identified by the curved shapes of these bacteria, and should be isolated before being visible to the naked eye. *Caulobacter* can be cultivated on ordinary peptone agar, if this medium is used soon after sterilisation. In a liquid peptone medium cultivation is not always successful.

A picture of a specimen from a liquid peptone medium in which *Caulobacter* occurred as a contaminant in a culture of *Ps. pyocyanea* was published by HOUWINK and VAN ITERSON (1950). Fig. 1 includes a smaller reproduction of this micrograph. It shows the principal characteristics of the species. The button-like holdfast at the base of the stalk is not a constant feature; it may be lacking. The bacterium is multiplying by transverse-binary fission. The apical cell grows a flagellum at its tip. One may suppose that this cell was about to come free.

Many cells moving swiftly may be observed in young cultures. On electron micrographs they turn out to possess a polar flagellum but no stalk as may be seen on critical examination of the relevant cells in fig. 2 and 11.

Once the bacterium has settled onto a substrate a stalk is grown, so that the body of the cell gets farther and farther away from the substrate. Often filaments radiate from the bases of the stalks (fig. 2 and 3). They may be comparable to those produced a.o. by *Pseudomonas pyocyanea* and *Escherichia coli* (*vide* HOUWINK and VAN ITERSON, 1950). Filament-growing seems connected with growth at a surface.

One problem is: which side of the bacterium clings to the substrate when the organism attaches itself? The micrographs of young *Caulobacter* show that it must be the flagellated end. With newly attached cells the flagellum is often found at the base of the stalk (fig. 4), but never at the opposite (free) end of the bacterium. The free end grows a flagellum only when fission of the cell seems not far off.

From the fact that the old flagellum is inserted at the base of the stalk it may already be inferred that the latter is part of the cell, instead of consisting of secreted material. The stalk does not look that way either. The main argument for the correctness of this conception is that in autolysed bacteria the stalk too looks more or less empty, with unidentified material irregularly distributed in the body of the bacterium and in the stalk alike (fig. 5).

Now that it has been established that the stalk is part of the cell, the conclusion seems warranted that the *Caulobacteriaceae* should be classified apart from the families that include bacteria with secreted stalks. Since the suborder was named after *Caulobacter*, this can be achieved in no other way than by elimination of these families from the suborder.

Jones (1905) was the first to cultivate in pure culture a bacterium which, according to its description and the photomicrographs published by her, fully answers the later definition of the *Caulobacteriaceae* Henrici et Johnson. A most striking feature of her photomicrographs are the rosettes formed by several tens of bacteria. She wrote: "This manner of grouping . . . is apparently effected by a uniform grouping of the descendants of a single cell and is in no sense an agglutinative phenomenon. The single polar flagellum of each organism is pointed toward an unseen center, the flagella appearing in stained preparations of rosettes as the radial arms of a windmill to which the vanes are attached". Obviously she took the stalks for flagella, though wondering at "the readiness with which the flagellum takes an ordinary stain". The true flagella she failed to observe.

Though small rosettes have often been found in the course of the present work (fig. 7), nothing as remarkable as Jones' pictures has been obtained. Jones may have been right in stating that the grouping is not an agglutinative phenomenon, but it does not follow that the cells of a rosette should be descendants of a single bacterium. Rosette formation may be due to many bacteria attaching themselves to one solid particle, which apparently is often very small. It is, however, quite possible that a central particle is lacking. In this case the bases of the stalks would have to be attached directly to one another. It cannot be determined as yet if this actually happens. If it does, the base of the stalk should be the only part of the *Caulobacter*'s surface fit for serving as a holdfast for another specimen of its kind, since *Caulobacter* does not seem able to attach itself to any other area of a kinsman's cell membrane.

Escherichia coli and *Pseudomonas pyocyanea* are like *Caulobacter* in that they do not serve as a holdfast for the latter genus, but *Bacillus subtilis* and *Bac. megaterium*, if cultivated in a diluted liquid peptone medium simultaneously with *Caulobacter*, may carry one to many stalked cells (fig. 8). The *Bacillus* does not immediately lose its motility. This proves that *Caulobacter* may settle on a living *Bacillus*. After a while the *Bacillus* lyses (fig. 9, 10, 11 and 12). In an earlier communication this has been depicted (Houwink, 1951). In a mixed culture in 0.1% peptone water *Bacillus* develops more rapidly than *Caulobacter*, but after a few days the latter are exceedingly numerous, whereas the number of viable *Bacillus* has decreased ten times or even more. A certain percentage of *Bacillus*, however, withstand the attack, for they are found never to be completely suppressed. *Caulobacter* does not seem to produce an antibiotic, for a cell-free filtrate of the medium of a mixed culture does not affect *Bacillus*.

These facts combined with the evidence from the micrographs suggested that *Caulobacter* was parasitic on *Bacillus*, and that the stalk acted as a sucking proboscis (HOUWINK, 1951). Though this supposition was attractive, it is now preferred to draw attention to the fact that somehow *Caulobacter* induces lysis of *Bacillus*. One gets the impression that also those individuals that have not got a victim of their own, thrive well on the medium which has been enriched by substances freed from the *Bacillus'* protoplasm.

The author wishes to express his thanks to Prof. Dr A. J. KLUYVER for his unfailing interest in this study. The work has been supported by the Netherlands Organisation for Pure Research (Z.W.O.).

Summary.

Arguments have been forwarded in favour of the view that the stalk of *Caulobacter* is part of the living cell. Accordingly the family *Caulobacteriaceae* Henrici et Johnson should be set apart from bacteria growing on stalks made of secreted material. This should be achieved by elimination of the latter from the suborder *Caulobacteriineae* Breed, Murray et Hitchens.

In a mixed culture of *Caulobacter* and *Bacillus* the former may induce lysis of the latter, after attaching themselves onto the surface of *Bacillus*. Apparently this favours the development of the *Caulobacter*.

Addendum.

In a recent paper (J. Bact. **68**, **194**) BOWERS, WEAVER, GRULÁ and EDWARDS (1954) criticised the description of the *Caulobacteriineae* Breed, Murray et Hitchens for the same reason as pointed out above (p. 50). They proposed that the description be changed so as to fit the genus *Caulobacter* Henrici et Johnson and the family *Caulobacteriaceae* Henrici et Johnson. The question whether or not BREED, MURRAY and HITCHENS were right in including, in the suborder, some families with quite a different type of stalk does not appear to have occurred to the authors. KANDLER, ZEHENDER and HUBER (1954), however, did discuss this question (Archiv Mikrobiol. **21**, 57). They advocated the exclusion, from the suborder, of the latter families for the same reasons as advanced by BISSET and by the present author.

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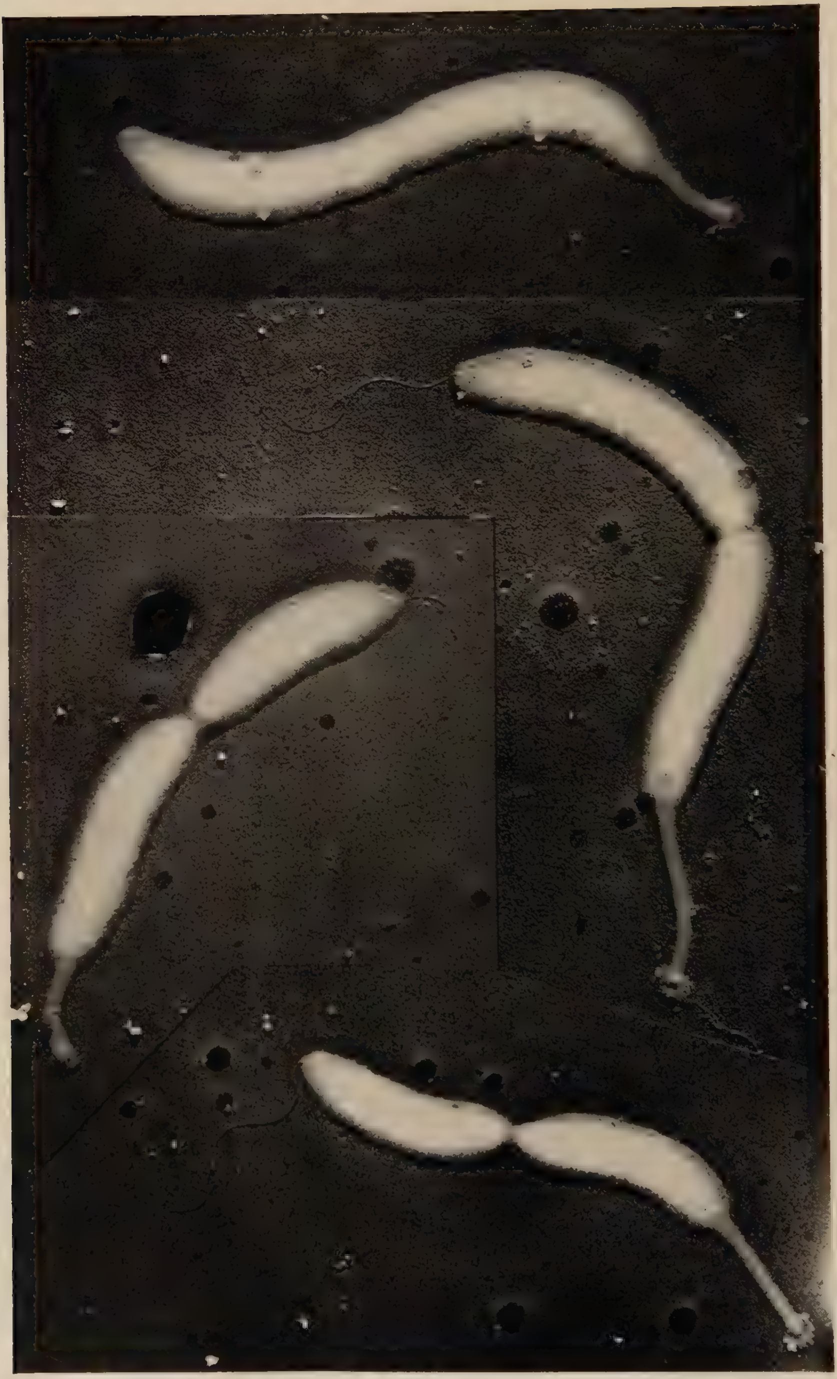

Fig. 1. *Caulobacter* as a contaminant in a culture of *Pseudomonas pyocyanea*. The bacteria have attached themselves to a collodion membrane floating on a diluted peptone medium. 15000 ×.

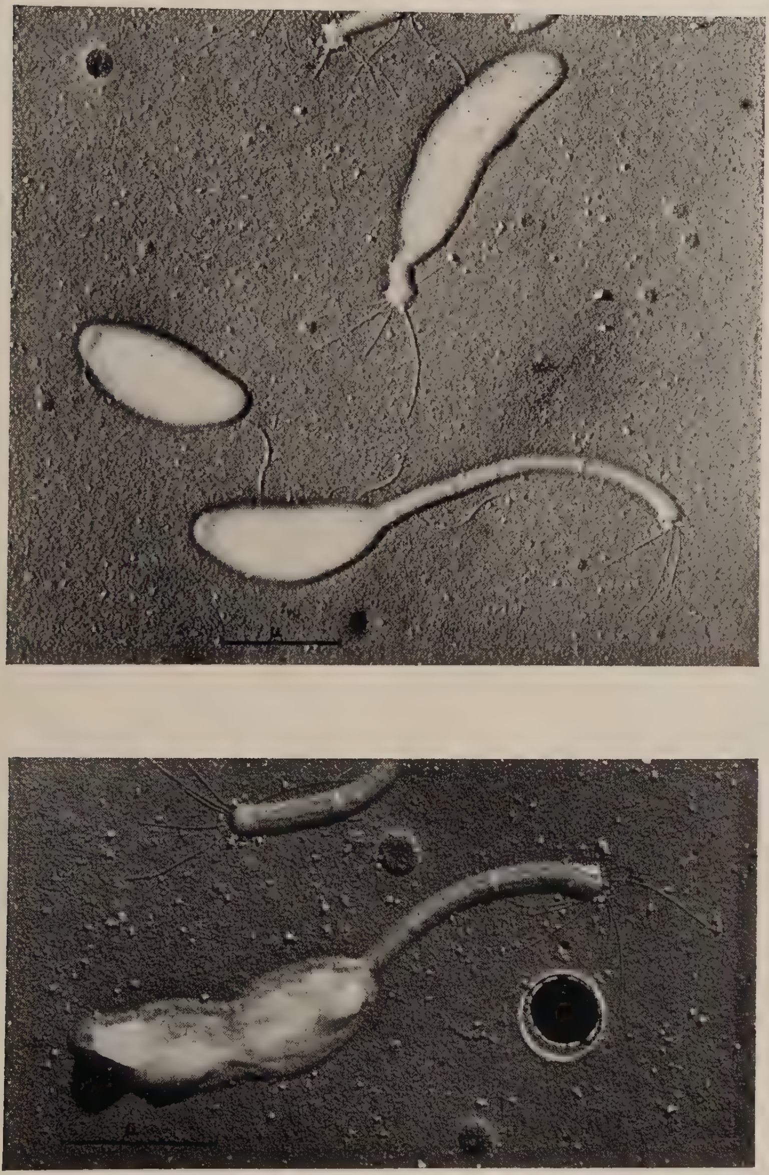

Fig. 2 and 3. *Caulobacter.* One cell has not yet grown a stalk but still possesses a flagellum (inserted at the left end of the cell). "Filaments" radiate from the bases of the stalks. Fig. 2, 15000 ×, Fig. 3, 20000 ×.

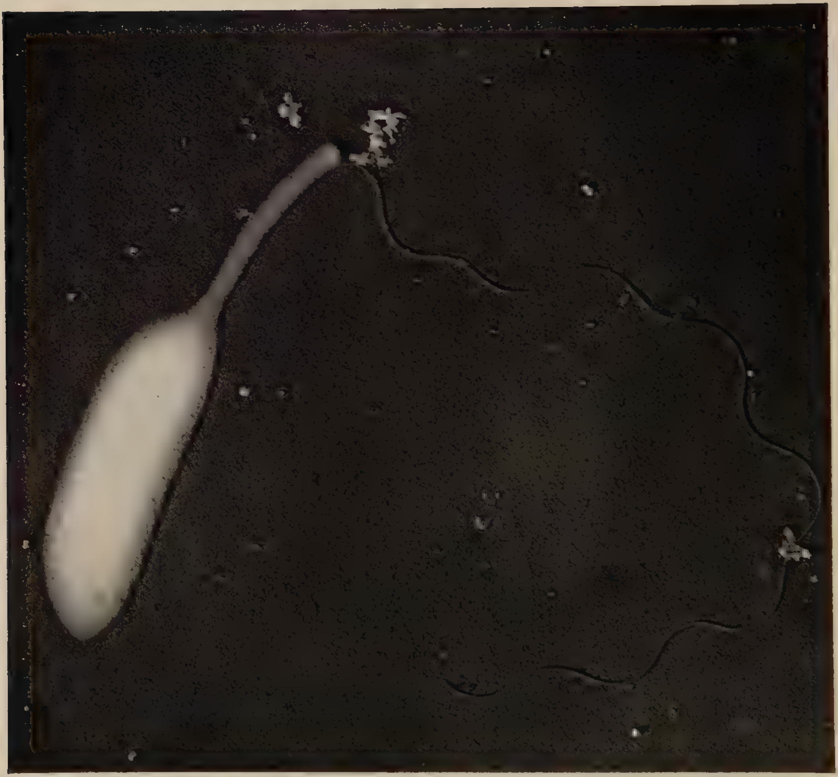

Fig. 4. Young *Caulobacter* with a flagellum inserted at the base of the stalk. ca. 20000 ×.

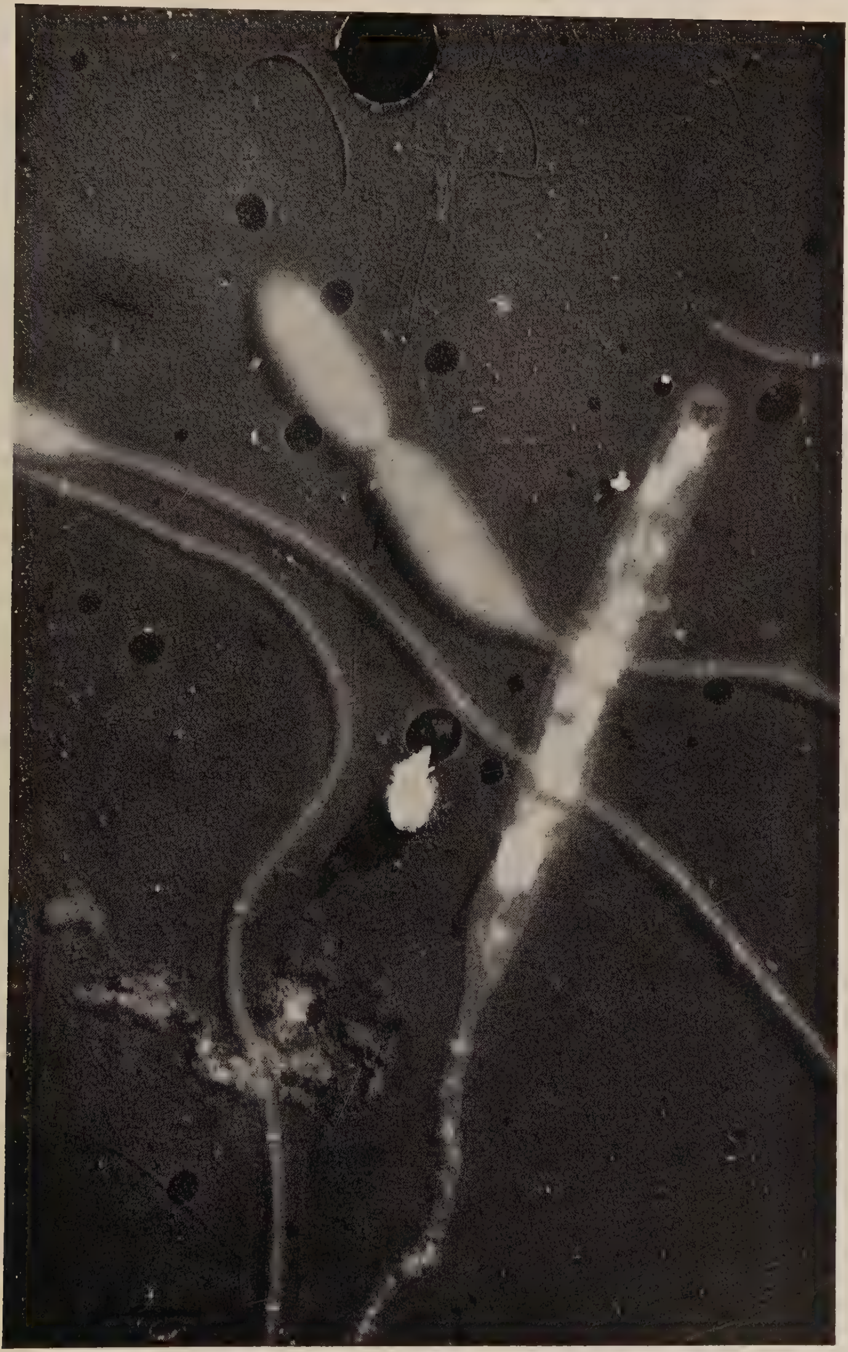

Fig. 5. *Caulobacter,* not in pure culture, presumably a different species. One cell has lysed; the stalk lysed too. 20000 ×.

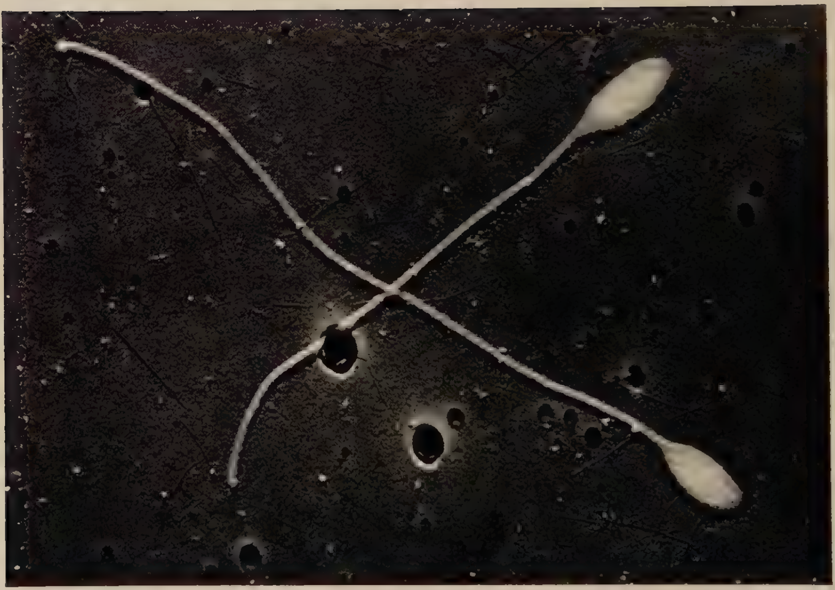

Fig. 6. *Caulobacter*, not in pure culture, maybe a third species, with small bodies and long stalks. 10000 ×.

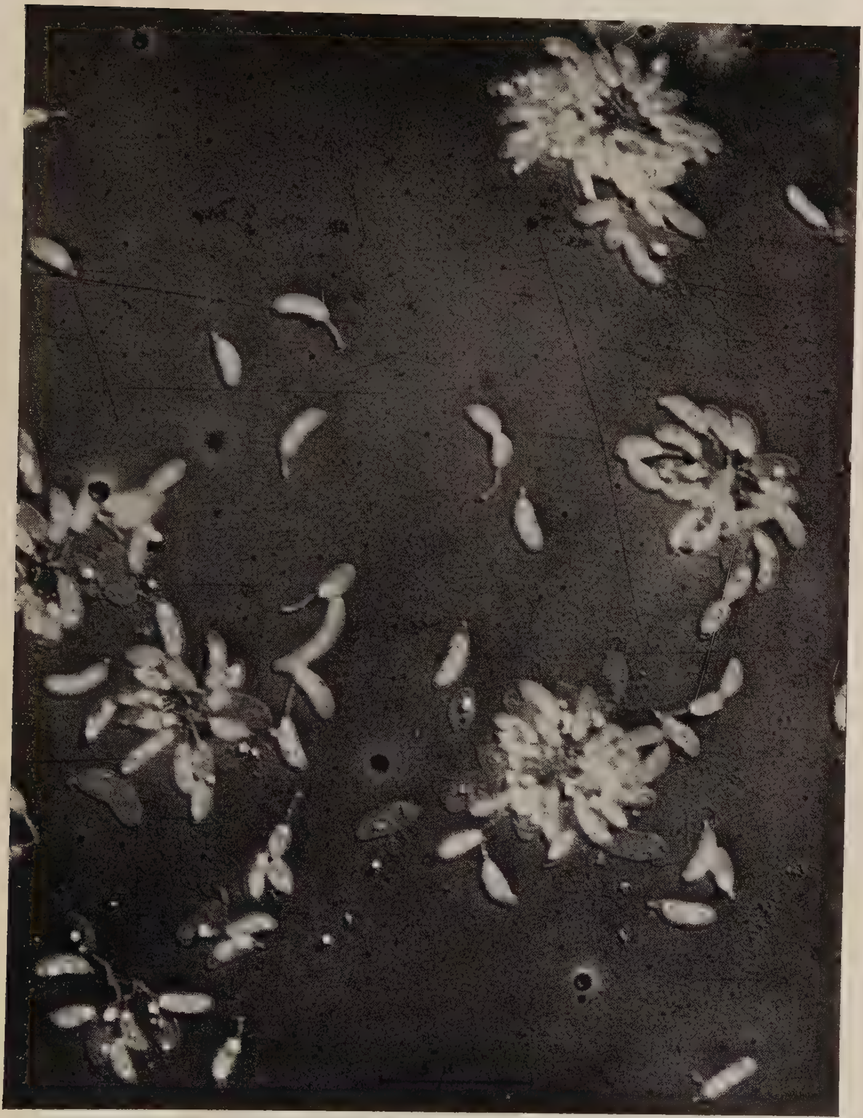

Fig. 7. *Caulobacter* rosettes in a pure culture. 4000 ×.

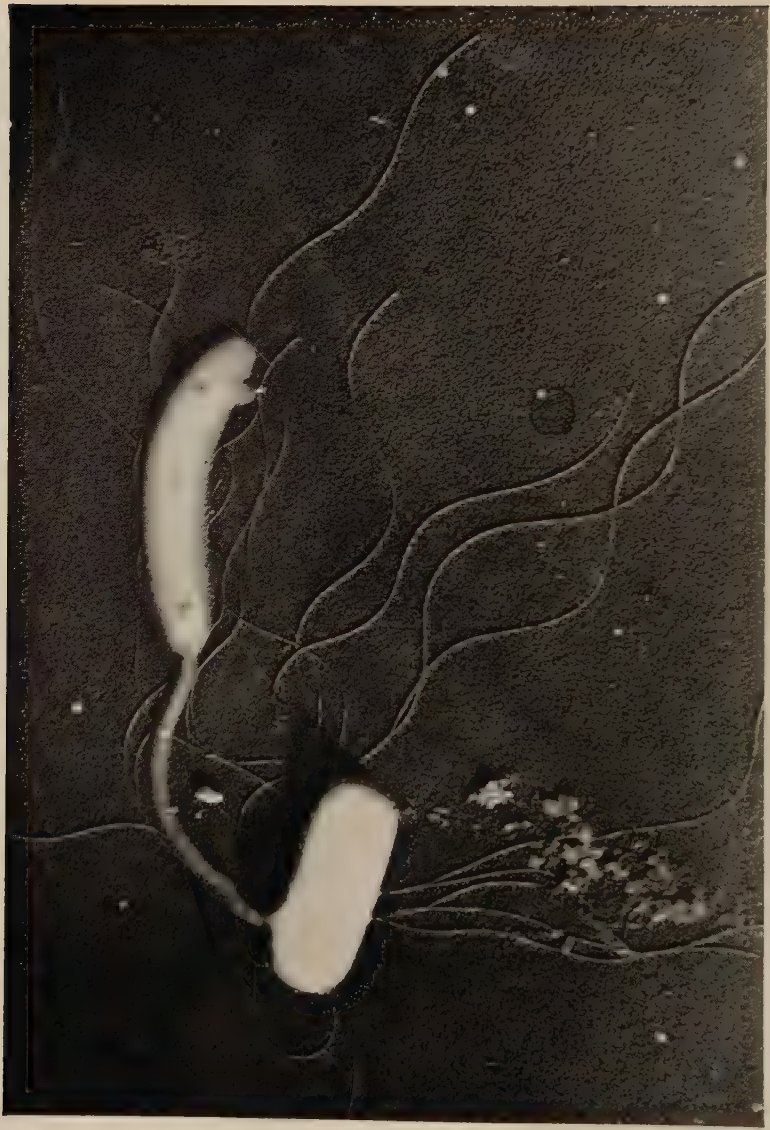

Fig. 8. *Caulobacter* attached to *Bacillus subtilis*. 15000 ×.

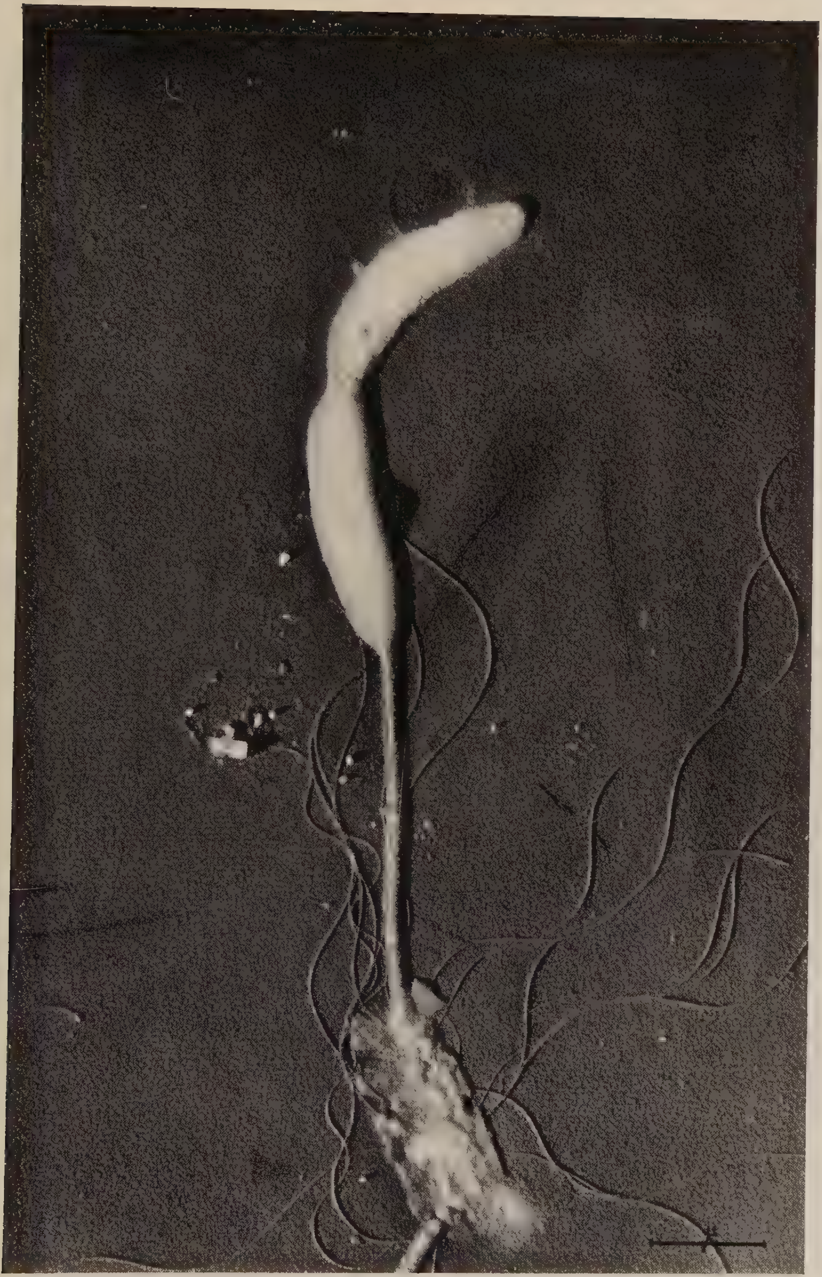

Fig. 9. *Caulobacter* attached to *Bacillus subtilis*. 15000 ×.

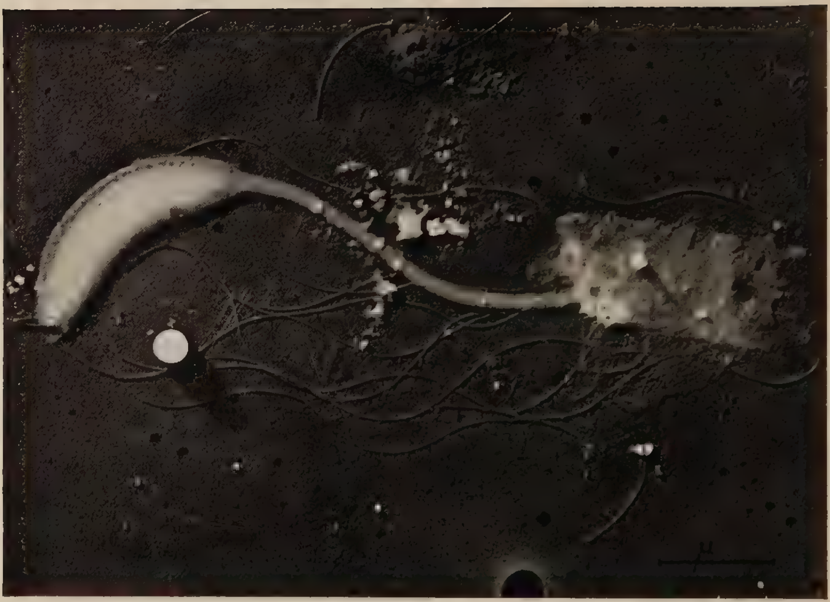

Fig. 10. *Caulobacter* attached to *Bacillus* spec. The globule is not a polystyrene particle! 15000 ×.

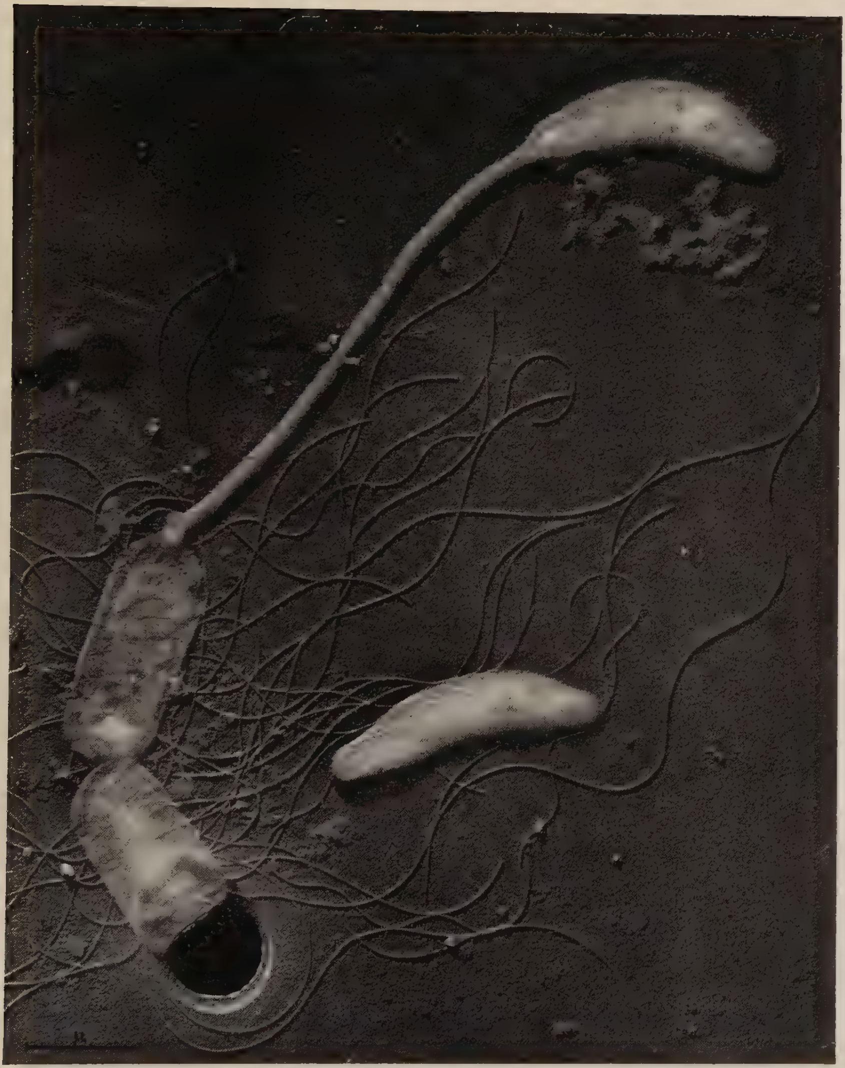

Fig. 11. *Caulobacter* attached to *Bacillus subtilis*. Next to this pair a young motile *Caulobacter*, with a flagellum inserted at its left tip. *Caulobacter* flagella have a shorter wavelength than those of *Bacillus*. 15000 ×.

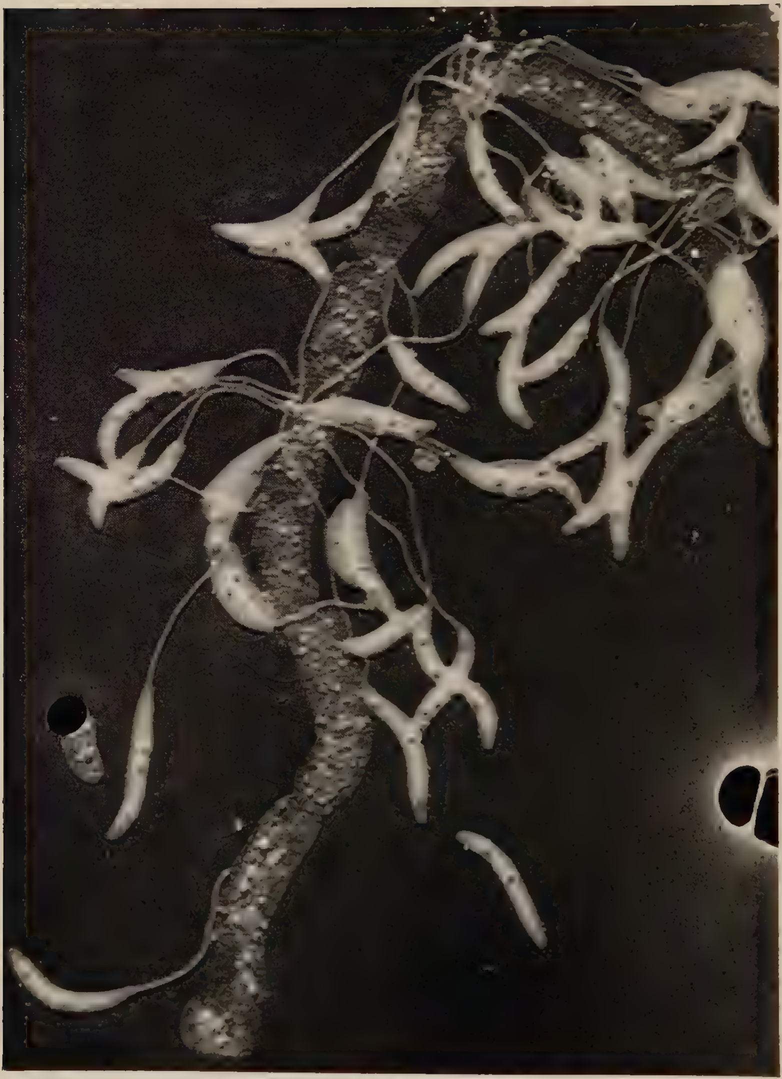

Fig. 12. *Caulobacter attached* to *Bacillus megaterium*. 6000 ×.

(Centraal Bureau voor Schimmelcultures, Baarn, Holland).

THE GENERA *ANIXIOPSIS* HANSEN AND *PSEUDEUROTIUM* VAN BEYMA

by

AMELIA C. STOLK

(Received April 9, 1954).

In this paper an account is given of the morphology and the taxonomy of three fungi, belonging to the genera *Anixiopsis* and *Pseudeurotium* of the family of the *Eurotiaceae*. Two of them, *Anixiopsis stercoraria* (Hansen) Hansen and *Pseudeurotium ovalis* n. sp., were encountered in the course of an investigation of fungi parasitizing the cysts of the Nematode *Heterodera rostochiensis* Wollenw. Both fungi were isolated by Dr P. A. VAN DER LAAN at Wageningen and were sent to the Centraal Bureau voor Schimmelcultures (C.B.S.), Baarn for identification. Related species were studied. *Anixiopsis japonica* Saito et Minoura proved to be synonymous with *Thielavia terricola* (Gilman et Abbott) Emmons. *Anixiopsis multispora* Saito et Minoura has been transferred to the genus *Pseudeurotium*. The conception of the genus *Pseudeurotium* was expanded and the genus was compared with related genera. The morphology and the development of the perithecia were described and figured. The perithecial initials of *Pseudeurotium ovalis* and *Anixiopsis stercoraria* consist of coiled hyphae, while those of *Pseudeurotium multisporum* are intercalary, swollen segments of the vegetative hyphae.

Living cultures of the studied species are maintained at the C.B.S. Dried cultures will be deposited in the Rijksherbarium, Leiden, Netherlands, in the Herbarium of the Royal Botanic Gardens, Kew and in the Herbarium of the Commonwealth Mycological Institute, Kew, England.

Anixiopsis stercoraria (Hansen) Hansen, Bot. Zeit. 55,1 27, 1897; syn. *Eurotium stercorarium* Hansen, Meddel. Naturhist. Foren, Kjöbenhavn, 310, 1876.

Anixiopsis stercoraria was described by HANSEN as *Eurotium stercorarium* in 1876. In 1897 he took up the study of this species again as the ascospores of the 21 years old specimen proved still viable and cultures were made. HANSEN then created for this fungus a new genus on account of the absence of an *Aspergillus* stage. HANSEN's fungus was isolated from fox dung in Jutland, Denmark, in 1901 MASSEE and SALMON found the fungus on owl excrements at Kew. Recently this species was isolated 10 times by Dr VAN DER LAAN from cysts of *Heterodera rostochiensis* Wollenw. The cysts had been collected from soil from potato fields near Huancayo, Sierre Central Peruana, Peru. Since no type material of *A. stercoraria* is available, it was only possible to compare the Peru isolates with the figures and the description of *A. stercoraria* by HANSEN. Although agreeing in most respects with this description, some differences were noted. HANSEN reported a rapid growth of his fungus on the common culture media but the Peru fungus on the contrary develops slowly. The ascospores of *A. stercoraria* are stated by HANSEN to be elliptical, 3 to 5 μ in length, while according to MASSEE and SALMON they are 4 μ in diameter; the ascospores of the Peru strains, however, measure 2.5 to 3 by 2 to 2.5μ. HANSEN described the occurrence of "Brutzellen", which are stated to be of different shape and size as mostly pear or sack shaped, 7 to 19 μ in length and frequently occurring in *Oidium* like chains. Probably the "Brutzellen" of HANSEN correspond with the chlamydospore-like structures present in the Peru fungi, although they differ somewhat in shape and appearance. The differences between the Peru strains and the description of HANSEN, however, are too slight to warrant at present the creation of a new species. A description of the morphology and the development of this interesting fungus will be given.

The fungus grows very slowly, attaining a diameter of 3.5 cm on malt agar, of 2 cm on cherry agar and of 3 cm on oatmeal agar enriched with a decoction of dung, in two months at room temperature. The cleistocarpous perithecia are produced abundantly on most media, especially so on rice and on oatmeal agar enriched with a decoction of dung. They are brown in young cultures, becoming grey-brown in age by the production of overgrowths of vegetative hyphae. The perithecia (fig. 1a) are found on the white, lanose aerial

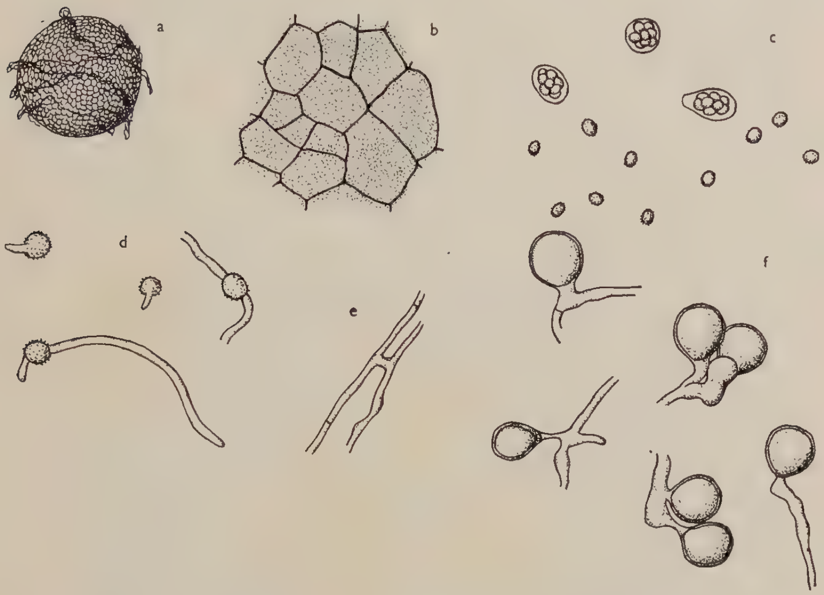

Fig. 1. *Anixiopsis stercoraria*. *a*. Perithecium, surrounded by vegetative mycelium. *b*. Outer wall cells of the perithecium. *c*. Asci and ascospores. *d*. Germinating ascospores. *e*. Anastomose between two hyphae. *f*. Chlamydospore-like structures of the mycelium. *a*: 80 ×; *b-f*: 830 ×.

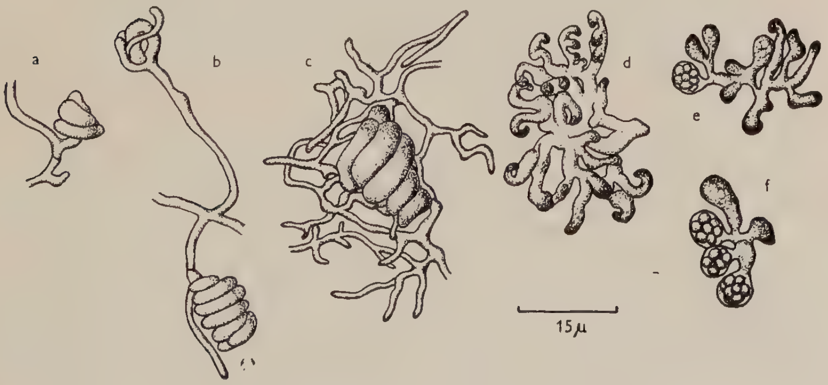

Fig. 2. *Anixiopsis stercoraria*.
a, b and *c*. Perithecial initials, consisting of coiled hyphae. *c*. Coil, surrounded by branching hyphae. *d, e* and *f*. Ascogenous hyphae, different stages in the development of the asci.

mycelium, they are globose and for the greater part glabrous, while the outer cell walls are polygonal (fig. 1b). The diameter of the perithecia is 140 to 220 μ. Microtome sections (fig. 3 and 4) showed them to have a stout wall, composed of about 5 layers of cells. These differentiated into an outer layer, 2 to 3 cells wide of thick-walled, red-brown cells and an inner zone of thin-walled cells. The centre of the perithecium consists of a thin-walled ground tissue in which numerous asci are embedded, each containing 8 ascospores. The asci are globose to subglobose, 6.5 to 7.5 by 5 to 6.5 μ and are deliquescing within the perithecium. The ascospores are subglobose to elliptical, 2.5 to 3 by 2 to 2.5 μ, conspicuously spinulose, hyaline when young and becoming pale yellow in age (fig. 1c). They are liberated by fracturing of the perithecium wall. Only a very small amount of the ascospores germinated in hanging drop cultures, containing oatmeal agar, malt or juice of cherries. Addition of a decoction of *Heterodera* cysts did not increase the percentage of germinating spores. From the fact that the ascospores of HANSEN's specimen still germinated after 21 years, it may be supposed that the ascospores have a long resting period before they germinate. The few spores which did germinate were swelling considerably, to about 5 μ, and produced 1 to 2 germtubes within 7 days at room temperature (fig. 1d).

No conidial stage has been observed. The mycelium, however, produces chlamydospore-like structures (fig. 1f), which are hyaline, thick walled, smooth and globose to somewhat pear shaped, 9 to 10 by 8.5 to 9 μ, they occur mostly terminal.

The vegetative hyphae are hyaline and about 1 to 1.5 μ wide, swellings may occur in the hyphae. Frequently anastomoses between two hyphae are found, mostly as H bridges (fig. 1e).

The perithecium develops from a hyphal coil (fig. 2a, b), which is often produced as a side branch from a vegetative hypha. The coiling hyphae, which are conspicuously swollen, stain deeply with cotton blue. No differentiated antheridial cell could be distinguished and no fusion has been observed between coiling hyphae. From this coil a nearly spherical structure originates. The hypha, from which the coiled side branch has arisen, gives off many branching filaments, which surround the coil and form the wall of the perithecium (fig. 2c). Microtome sections of young perithecia show the formation of the peridial wall by a thickening of the walls of the outer cell layers. Within these wallcells a mass of hyaline pseudoparenchymatous tissue is formed, in the centre of which some deeply staining hyphae are found, the ascogenous hyphae. The asci are abundantly produced as side branches from the ascogenous hyphae (fig. 2d, e and f). The development of asci continues until the centre of the perithecium is filled and while increasing in number, the asci become interpolated between the cells of the ground tissue (fig. 3). Ultimately this ground tissue, the ascogenous hyphae and the asci disappear and the perithecium is completely filled with the pale yellow ascospores.

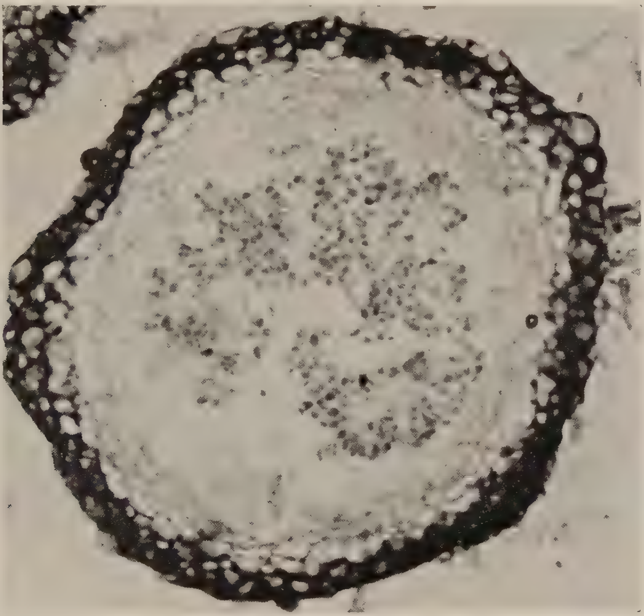

Fig. 3. *Anixiopsis stercoraria*.
Microtome section ¹) through a perithecium, × 400.

The different Peru strains of this fungus received from Dr van der Laan agree in all characteristics. Experiments carried out by Dr van der Laan have proved that this fungus is capable of parasitizing the cysts of *Heterodera rostochiensis* Wollenw.

The Peru fungus was compared with the description of *Mycogala*

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¹) The author wishes to thank Mr. P. den Houter for the preparation of the microtome sections and the photographs.

marginata Crooks (1935). Unfortunately no authentic material of this species was available. According to the description it differs from *A. stercoraria* in the character of the ascospore wall and in the conidial stage. Both species are not identical but perhaps closely related.

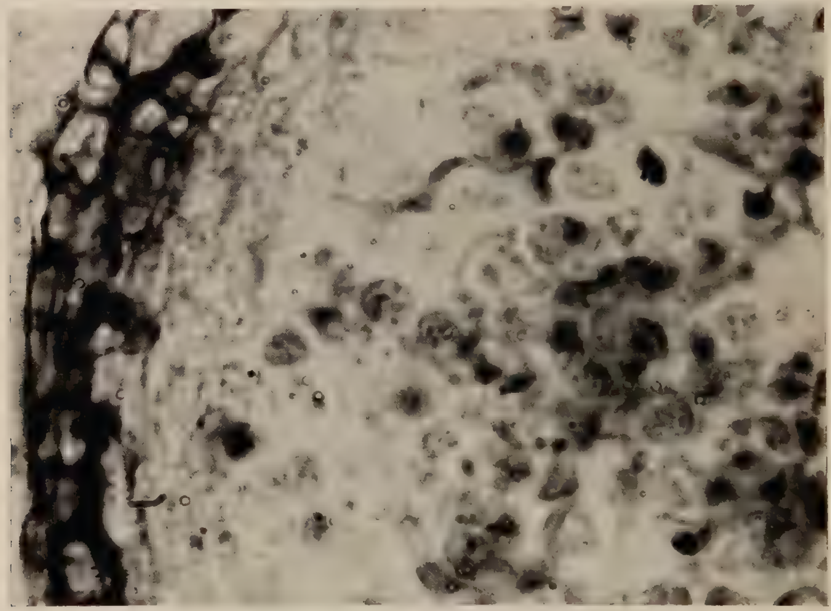

Fig. 4. *Anixiopsis stercoraria.*
Microtome section through a part of a perithecium, showing young asci with ascospores, × 1500.

Saito and Minoura (1948) described two species of *Anixiopsis*, *A. japonica* and *A. multispora*. Strains of both species have been received through courtesy of the Nagao Institute, Japan, the Institute for Fermentation, Osaka, Japan and the American Type Culture Collection, Washington D.C.

Examination of the type strain of *A. japonica* Saito et Minoura, N.I. 2002, however, proved it to be identical with *Thielavia terricola* (Gilman et Abbott) Emmons. The ascospores, measuring 14 to 16 by 7 to 9 μ, have one apical pore. The fungus only produces ripe perithecia when cultivated at 25° C. *Th. terricola* was described in 1927 by Gilman and Abbott as *Coniothyrium terricola* and in 1930 it was transferred to the genus *Thielavia* by Emmons. On reasons of priority, therefore, *A. japonica* must be considered as a synonym of *Thielavia terricola* (Gilman et Abbott) Emmons.

Examination of the type strain N.I. 2003 of *A. multispora* Saito et Minoura, made it clear that this fungus does not belong to the genus *Anixiopsis* Hansen either. According to SAITO and MINOURA (1948), fungi belonging to the family of the *Eurotiaceae* and not developing a conidial stage, should be placed in the genus *Anixiopsis*. The classification of the *Ascomycetes*, however, is based on the morphology of the perithecia, asci and ascospores and not on the occurrence of a conidial stage. SAITO and MINOURA's species has brown, smooth ascospores, such as occur in the genus *Pseudeurotium*. The genus *Anixiopsis* on the contrary is characterized by spinulose, hyaline to subhyaline ascospores. It seems better therefore to transfer *A. multispora* to the genus *Pseudeurotium*.

As this interesting fungus is somewhat concealed in the literature because it was described in Japanese, a short re-description of this species based on observation of the type strain will follow.

Pseudeurotium multisporum (Saito et Minoura) n. comb.
syn. *Anixiopsis multispora* Saito et Minoura, Journ. of Ferm. Techn. **26**, 3, 1948.

Colonies of this fungus on oatmeal agar in Petri dishes grow rapidly, attaining a diameter of 7 cm in 3 weeks at room temperature. The submerged mycelium is light ochraceous-salmon (RIDGWAY, 1912), when growing in daylight, while uncoloured when cultivated in the dark. Cleistocarpous, dark brown to black perithecia, 120 to 280 μ in diameter are abundantly produced on the surface of the agar, but also in the culture medium, growing in somewhat indistinctly concentric zones. The perithecia have a thin wall of polygonal cells (fig. 6a). Microtome sections showed this wall to be composed of a single layer of cells (fig. 5).

The outer walls and especially the side walls of these peridial cells are thickened, the inner walls, however, remain thin. The centre of the perithecium consists of a ground tissue, in which numerous asci are embedded. The asci are produced as side branches from the ascogenous hyphae and also apically on these hyphae (fig. 6b). They are globose to subglobose and measure 15 to 20 by 10 to 14 μ (fig. 6c). Each ascus contains about 32 ascospores, which are smooth, grayish olive and cylindrical with rounded ends and sometimes very slightly curved. They measure 5 to 6 by 3 to

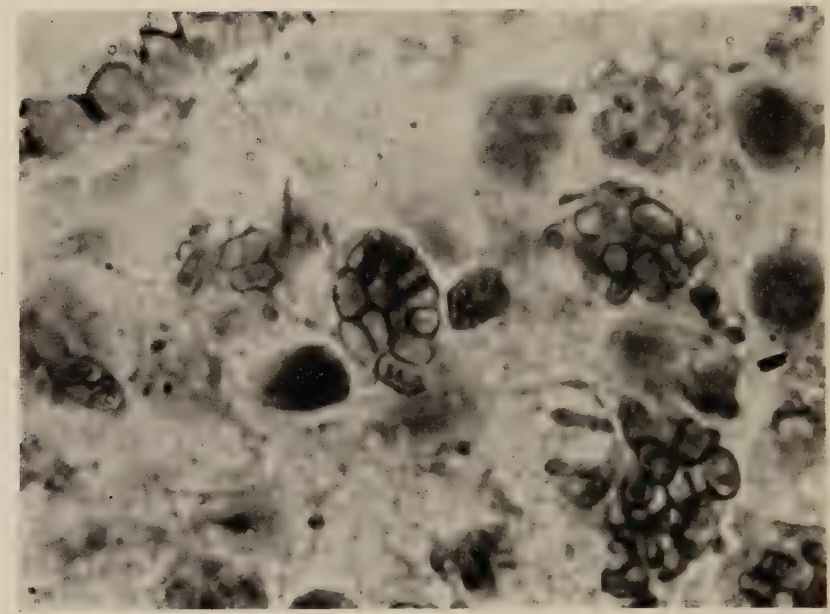

Fig. 5. *Pseudeurotium multisporum.*
Microtome section through a part of a perithecium, showing asci with many ascospores and the typical thickening of the side and outer walls of the peridial wall cells, × 1500.

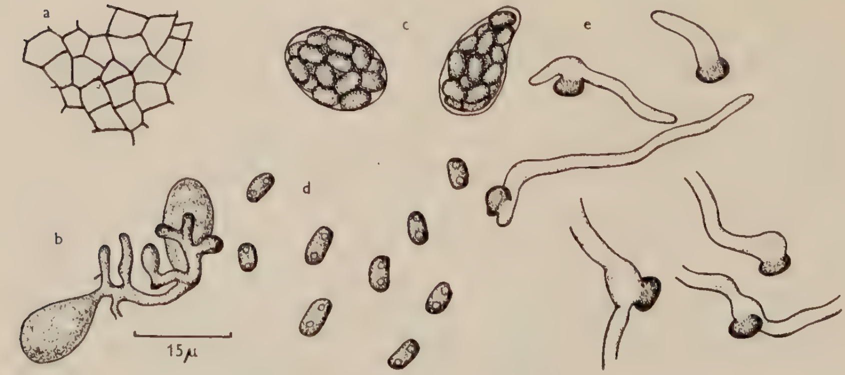

Fig. 6. *Pseudeurotium multisporum.*
a. Polygonal wallcells. *b.* Ascogenous hyphae with young asci. *c.* Asci. *d.* Ascospores. *e.* Germinating ascospores.

3.2 μ and generally contain 2 oildrops (fig. 6d). The ascospores germinate readily. In 24 hours at 25°C. one germtube develops, originating from the side of the ascospore (fig 6e), the broadened proximal part of this germtube produces one or sometimes two side branches. No conidial stage has been observed.

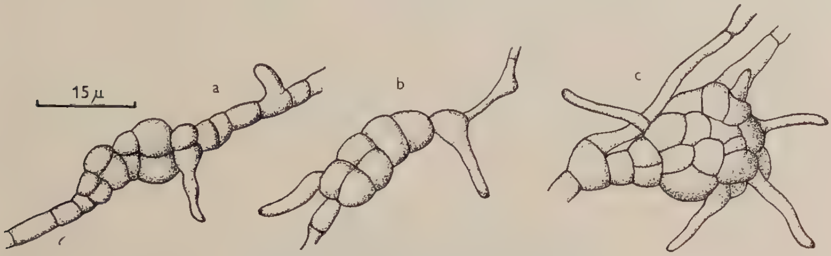

Fig. 7. *Pseudeurotium multisporum.*
a, *b* and *c*. Perithecial initials.

The perithecium does not develop from a hyphal coil such as occurs in the other two species dealt with in this paper, but it originates from intercalary segments of the mycelium, which become swollen and divide into short cells. The perithecial initials can already be found in one week old cultures, they show characteristic staining reactions (fig. 7). Side branches develop and many crosswalls are formed in these swollen cells, thus producing a solid, spherical mass of pseudoparenchymatous tissue with little or no differentiation, except for the somewhat thicker walls of the cells of the outer layer. In the centre of the pseudoparenchymatous tissue ascogenous hyphae can be found. The mature perithecium is completely filled with the brown ascospores, since ground tissue, ascogenous hyphae and asci disappear.

In the vegetative hyphae coils are often found. The cells of these coils show not the characteristic staining reaction and they are not to be confused with perithecial initials.

Pseudeurotium ovalis n. sp.

This fungus[1] was isolated by Dr van der Laan in March 1953 from cysts of *Heterodera rostochiensis*. The cysts were found in fine sandy soil, which had been collected on the island of Jersey (Channel Islands).

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[1] Recent experiments by Dr van der Laan have proved that this fungus also attacks the cysts of *Heterodera rostochiensis* Wollenw.

Colonies of this fungus on oatmeal agar in Petri dishes grow slowly, attaining a diameter of 9 cm in 6 weeks at room temperature. The cultures develop alternating zones with dark brown to black perithecia about 1 to 2 mm wide and zones only consisting of white cottonlike hyphae (fig. 8). The reverse of young colonies is light viridine yellow, becoming olive-buff in older cultures (Ridgway, 1912).

The fungus readily produces cleistocarpous perithecia on most media, especially on oatmeal agar. The perithecia are found, not only on the surface, but also within the upper part of the culture medium. They are globose, glabrous, brownish to almost black, 90 to 180 μ in diameter (fig. 9a). When the perithecium is ripe, it fractures by splitting (fig. 9b). The wall of the mature perithecium consists of only one layer of polygonal, dark brown and thick-walled cells (fig. 9c and 11). The evanescent asci, containing 8 ascospores, are globose to elliptical, 7.5 to 9 by 6.5 to 8 μ. The young ascospores are hyaline, the mature spores, however, are olive, smooth, elliptical, 5.5 to 6 by 3.5 to 4 μ, the rounded ends have thickened walls (fig. 9d).

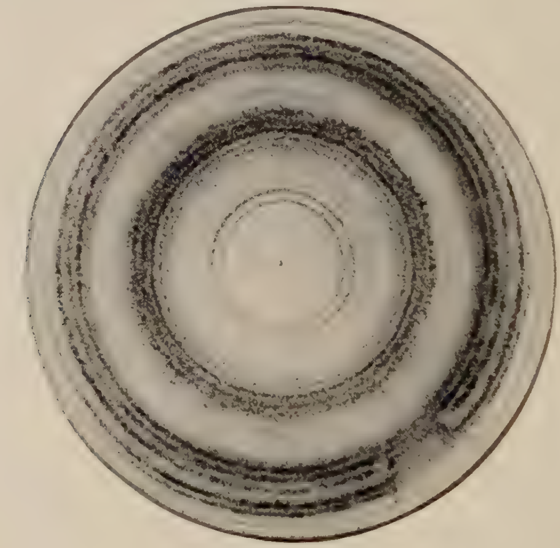

Fig. 8. *Pseudeurotium ovalis.*
Culture on oatmeal agar in a Petri dish, showing concentric zones.

No germpore has been observed. Only a small percentage of the ascospores germinate in a hanging drop culture, containing oatmeal agar, malt or a decoction of cherries. The amount of germinating ascospores did not increase by bringing the ascospores into a decoction of the *Heterodera* cysts. The germinating ascospores produce one germtube in 24 to 48 hours at 25°C., mostly this tube originates from the side of the ascospores, somewhere in between the two thickened ends (fig. 9e). The germtube produces near the germinating sporebody one to two side branches and in three days old cultures conidia are already formed along the hyphae.

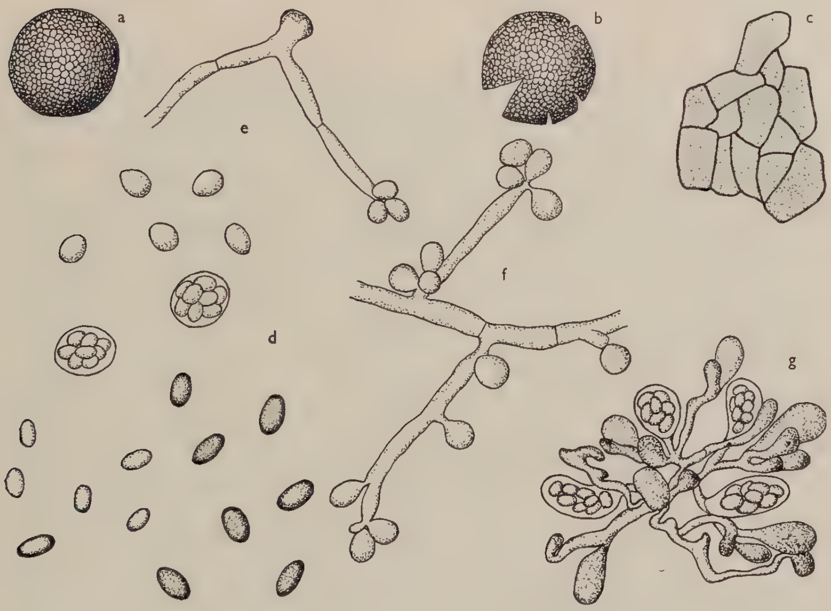

Fig. 9. *a* and *b*: 80×; *c-g*: 830 ×. *Pseudeurotium ovalis*.
a. Perithecium. *b*. Fracturing perithecium. *c*. Polygonal wallcells. *d*. Asci, young and mature ascospores. *e*. Germinating ascospore, the young hypha already produces conidia. *f*. Sporotrichum stage. *g*. Ascogenous hyphae with asci in different stages of development.

The conidial stage of the fungus shows the characteristics of the genus *Sporotrichum* Link ex Fries. Conidia develop along the hyphae and on conidiophores, they are produced on small teeth, usually 3, 4 or more together in rosettes. The length of the conidiophore ranges from 20 to 45 μ, its diameter is about 3 to 4 μ. The hyaline, subglobose to pearshaped conidia measure 4.5 to 6 by 3.5 to 4.5 μ (fig. 9e and f).

Mycelium and conidiophores are hyaline. The diameter of the hyphae is about 2.5 μ. Between the hyphae anastomoses often occur, mostly as H bridges.

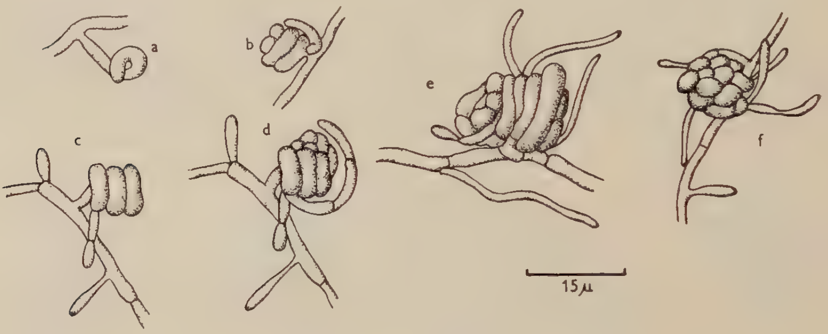

Fig. 10. *Pseudeurotium ovalis.*
a—f. Development of the perithecium from a hyphal coil, *c* and *d* are two successive stages in the development of the perithecium, studied in a hanging drop culture.

The perithecium develops from a hyphal coil (fig. 10). From the base of the primary coil side branches appear, which coil round one another to produce a nearly spherical structure. No fusion has been observed between the coiling hyphae. Often non-coiling side branches are produced as well. Microtome sections of young perithecia showed an early development of the perithecium wall, by thickening of the wallcells. In the centre of the pseudoparenchymatous tissue the asci are formed as branches from the ascogenous hyphae (fig. 9g and 11). Finally ground tissue, ascogenous hyphae and asci disappear.

The present fungus has been compared with related forms in the genera *Cephalotheca* (Fuckel, 1871), *Allescheria* (Saccardo et Sydow, 1899) and *Pseudeurotium* (van Beyma, 1937).

In 1889 Ch. Richon described *Cephalotheca palearum.* The spores are stated to be fuscous, subglobose, 4 μ large. The description and the figures of ascogenous hyphae and spores do not agree with those observed on the present species. Although our fungus seems to be related to Richon's species, it was not possible to identify it with *Cephalotheca palearum.*

The fungus under consideration differs from *Allescheria boydii* Shear (Shear, 1922; Emmons, 1944) in the shape, colour and germination of the ascospores and in the conidial stage.

In several respects the present fungus resembles *Pseudeurotium zonatum* van Beyma (1937). Both fungi produce cleistocarpous, glabrous and brown perithecia in conspicuous zones, they have smooth, brown ascospores and the conidial stage of both species is a *Sporotrichum*. Our fungus, however, differs from *P. zonatum* in producing elliptical ascospores 5.5 to 6 by 3.5 to 4 μ, whereas the ascospores of *P. zonatum* are globose, 3 to 3.5 μ. It seems best therefore to consider the present fungus as a new species of the genus *Pseudeurotium*.

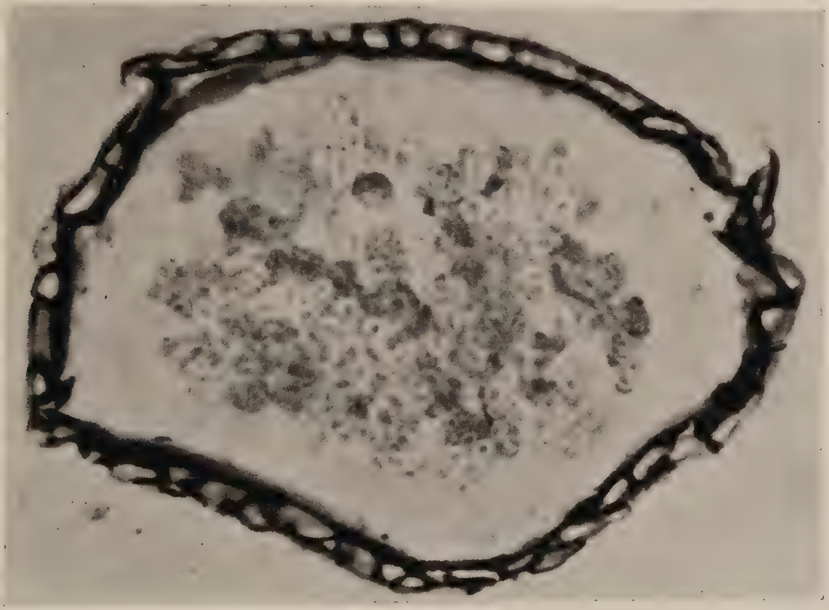

Fig. 11. *Pseudeurotium ovalis*.
Microtome section through a perithecium, containing asci and ascospores. The wall consists of one layer of thick walled cells. × 1000.

The genus *Pseudeurotium* van Beyma was based on a single species, *P. zonatum*. The diagnosis of this genus should be slightly expanded to include the above described fungi as well.

Pseudeurotium should be placed in the *Phaeosporeae* of the family of the *Eurotiaceae* (Clements and Shear, 1931), near the genera

Cephalotheca Fuckel (1871) and *Thielavia* Zopf (1875). It differs from *Cephalotheca* in the structure of the perithecium wall.

In *Cephalotheca* the perithecia are fracturing at maturity along well defined sutures, which divide the wall in polygonal plates (Chesters, 1935). Besides the perithecium wall of *Cephalotheca* being rather thick, it consists of 5 or more layers of thick walled cells. In these thickened walls very thin parts occur, the sutures. In the species of *Pseudeurotium*, however, no well defined sutures have been observed and the perithecium wall consists of only one layer of cells with evenly thickened walls. The genus *Thielavia* differs from *Pseudeurotium* by its ascospores, which have one to two germpores, whereas in *Pseudeurotium* no germpores are found.

Diagnosis of the genus *Pseudeurotium* van Beyma.

Perithecia simple, astomous, spherical, glabrous and carbonaceous, not fracturing along well defined sutures. Perithecium wall mostly consisting of only one layer of cells. Asci produced as side branches from ascogenous hyphae, globose to elliptical, containing 8 or many ascospores. Ascospores conglobate, continuous, fuscous, smooth, globose or elliptical, without germpores. A conidial stage may occur.

Pseudeurotium ovalis sp. n.

Perithecia spheroidea, astomata, atrobrunnea, 90—180 μ diametro, formata per zonas concentricas, aut submersa in substrato aut in superficie eius. Paries perithecii ex uno ordine cellularum polygoniarum brunnearum parietibus crassis. Asci spheroides vel ellipsoides, 7,5—9 μ longi, 6,5—8 μ lati, mucosi, 8-spori.

Ascosporae ellipsoides, continuae, primo hyalinae, maturae brunneae, 5,5—6 μ longae, 3,5—4 μ latae, parietibus levibus in parte rotunda crassatis. Pori desunt. Status imperfectus est *Sporotrichum*. Conidia formata in locis parvulis eminentibus immediato hyphis aut conidiophoribus. Conidiophora 20—45 μ longa, 3—4 μ lata. Conidia ellipsoidea vel piriforma, glabra, hyalina, 4,5—6 μ longa, 3,5—4,5 μ lata.

Mycelium hyalinum, ex hyphis 2,5 μ diam.

Hab.: in cystis *Heteroderae rostochiensis* Wollenw., insula Jersey, Brittannia, leg. P. A. van der Laan.

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FOUR NEW *SALMONELLA* SEROTYPES ISOLATED IN CURACAO

by

P. R. EDWARDS, F. J. RUTTEN and **ALMA C. McWHORTER**

(Received April 5, 1954).

Among a number of *Salmonella* cultures isolated from residents of the Netherlands Antilles and from their environment, the four new serotypes described below were found. All of the cultures possessed the usual cultural and biochemical properties of the genus *Salmonella*. None produced indol while all were methyl red positive and Voges-Proskauer negative. None fermented lactose, sucrose, or adonitol and none hydrolyzed urea. All fermented glucose, rhamnose, xylose, arabinose, maltose, trehalose and mannitol promptly with the production of gas. All produced acid in Jordan's tartrate agar. The cultures differed in their reactions in the remaining biochemical tests employed.

Salmonella serotype 4225—53, 4,5,12: g,m,s. [1]).

This type was represented by only one culture isolated from a stool of a two months old female child affected with gastroenteritis. The organism produced H_2S and grew in Simmons' citrate agar. Salicin and raffinose were not fermented and gelatin was not liquefied. Dulcitol, sorbitol and inositol were fermented promptly.

On serologic examination the organism was found to be a member of group B of the genus and to possess O antigens 4,5,12. In absorption tests it completely exhausted agglutinins from *S. typhimurium* O serum. In H agglutination tests the bacterium was agglutinated to the titers of *S. enteritidis* (g,m) and *S. montevideo* (g,m,s) serums and to a lesser degree by H serums derived from related forms. It was agglutinated by single factor serums *m* and *s* but not by *f*, *p*, *q*, *t*, or *u* serums. In absorption tests the organism removed all H agglutinins from *S. montevideo* serum.

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[1]) In accordance with a recent decision of the Enterobacteriaceae Subcommittee of the International Association of Microbiologists, the serotypes described here are designated only by antigenic formula and not by name.

Salmonella serotype 4624—53, 6,7: z_4,z_{24}.

In addition to the type culture which was isolated from the water of a cistern at a school in Kralendyk, Island of Bonaire, a second culture (4638—53) was isolated from the stools of an adult male affected with diarrhea and fever. Although the man lived on the Island of Bonaire, no epidemiologic connection between the cultures was established. The cultures produced H_2S and fermented sorbitol promptly. Simmons' citrate agar was utilized slowly by 4624—53 but not by 4638—53. Salicin was fermented slowly and gelatin was liquefied after 20 to 30 days at 25° C. Dulcitol, inositol and raffinose were not attacked.

The cultures were members of group C_1 and were agglutinated to the titer of *S. thompson* O serum. In absorption tests the organisms reduced the titer of the serum by 95 per cent. In H agglutination tests the cultures were flocculated by *S. cerro* (z_4,z_{23}) and *S. düsseldorf* (z_4,z_{24}) serums. They were agglutinated by single factor z_{24} serum but not by z_{23} serum. In absorption tests they removed all H agglutinins from *S. düsseldorf* serum.

Salmonella serotype 4626—53, 28: d-1,7.

The type culture was isolated from the stool of a five weeks old male child affected with diarrhea and fever. Blood and mucous were present in the stools. A second culture of the same type (4694—53) was isolated from the infant two weeks later, at which time diarrhea and fever still persisted but blood or mucous was not observed. The organism produced H_2S and utilized Simmons' citrate agar. Dulcitol was fermented promptly but salicin, inositol and raffinose were not attacked and gelatin was not liquefied. Sorbitol was not fermented by strain 4626—53 but culture 4694—53 produced acid and gas from sorbitol after 3 days incubation.

The organism was agglutinated to the titer of *S. pomona* O (28) serum but, in absorption tests, a residue of agglutinins amounting to 20 per cent of the titer was left. The H antigens of the culture were diphasic and phase 1 was flocculated to the titer of *S. typhi* H (d) serum. In absorption tests the organism reduced the titer of the serum from 20,000 to 100. Phase 2 of 4626—53 was agglutinated actively by serums derived from 1,2; 1,5; 1,6; and 1,7 phases of the genus. When tested with single factor serums 2, 5, 6, and 7, agglutination occurred only in factor 7 serum. The organism did not remove all agglutinins for the homologous culture from S. *madelia* phase 2 (1,7) serum but did remove from the serum all agglutinins for phase 2 of S. *altendorf* (1,7).

Salmonella serotype 4693—53, 38: r-1,6.

This serotype was represented only by one culture isolated from the stool of a 49 year old male food handler who gave no history of symptoms of recent enteric infection. The organism failed to produce H_2S as judged by its inability to blacken triple sugar iron agar (Difco) or lead acetate papers suspended over cultures in 2 per cent Bacto peptone water. Growth occurred promptly in Simmons' citrate medium. Dulcitol was fermented promptly. Acid and gas were produced from raffinose after 4 days and from salicin after 25 days' incubation. Inositol was not attacked and gelatin was not liquefied.

The culture was agglutinated to the titer of, and removed all agglutinins from, S. *inverness* O (38) serum. The H antigens were diphasic and phase 1 was agglutinated to the titer of S. *rubislaw* phase 1 (r) serum. In absorption tests 4693—53 removed all agglutinins from the serum. Phase 2 was agglutinated by 1,2; 1,5; 1,6; and 1,7 serums but when tested with single factor serums 2, 5, 6, and 7, it was agglutinated only by factor 6 serum. It agglutinated to the titer of S. *anatum* phase 2 (1,6) serum but in absorption tests it left a residue of agglutinins for the homologous strain which amounted to 2 per cent of the original titer of the serum.

Summary.

The following new *Salmonella* serotypes were found in Curaçao:

a) 4225—53 — 4,5,12: g,m,s — from the stool of a two months old child affected with gastroenteritis.

b) 4624—53 and 4638—53 — 6,7: z_4,z_{24} — from the water of a school cistern and from the stool of an adult male affected with diarrhea and fever.

c) 4626—53 — 28: d-1,7 — from the stool of a five weeks old child affected with diarrhea and fever.

d) 4693—53 — 38: r-1,6 — from the stool of a normal adult food handler.

(Institut Agronomique, Université de Louvain, Belgique).

ETUDE DES PREMIÈRES ÉTAPES DE LA DÉGRADATION DU GLUCOSE CHEZ *PENICILLIUM BREVI-COMPACTUM*

par

PAUL GODIN [1])

(Reçu le 26 Mai 1954).

Les résultats de recherches antérieures (GODIN, 1953-1954) sur le métabolisme ternaire de *P. brevi-compactum* ont notamment permis de présenter deux hypothèses de travail concernant la dégradation du glucose par cette moisissure. La première hypothèse, avancée à la suite de la mise en évidence par chromatographie, des produits du métabolisme du glucose que voici: acides gluconique, 2-cétogluconique et glucuronique, ribose, arabinose, arabitol ou adonitol et i-érythritol, suppose que l'oxydation directe du glucose est réalisée par la moisissure. La seconde hypothèse, basée sur la découverte du fructose et du glycérol parmi les produits de transformation du glucose par la moisissure, suppose l'existence de la glycolyse.

Cette note décrit les recherches entreprises dans le but de vérifier la réalité de ces processus. Les résultats obtenus permettent notamment d'avancer certaines précisions sur le déroulement de l'oxydation directe chez *P. brevi-compactum* et sur la relation existant entre ce processus et la glycolyse.

MÉTHODES EXPÉRIMENTALES.

Conditions de culture.

La moisissure est ensemencée sur 200 ml de milieu Czapek-Dox, contenus dans un erlenmeyer de 500 ml de capacité. Après développement en surface du mycélium, à 25°C., on substitue au milieu initial, suivant la technique décrite par Foster (1944), 100 ml de la solution à expérimenter. On fait ensuite incuber la moisissure, toujours à 25°C., au contact de cette nouvelle solution nutritive.

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[1]) Chargé de Recherches du Fonds National de la Recherche Scientifique; Adresse actuelle: Laboratoire de la Sté. Steinbach et Cie., Malmédy, Belgique.

Les solutions substituées renferment du glucose ou de l'acide gluconique, et des quantités croissantes de l'inhibiteur de la desmolyse dont on veut étudier l'effet sur le métabolisme de la moisissure. Les inhibiteurs expérimentés sont: l'acide iodoacétique, le fluorure sodique, le chlorure cuivrique, l'azide sodique et le sulfite de calcium.

Méthodes d'analyse et de dosage.

La glucose de la solution de métabolisme est dosé polarimétriquement. Dans les solutions substituées d'acide gluconique, on dose cet acide par titrimétrie: on ajoute à la solution un excès connu de NaOH N/10, porte à ébullition, refroidit et titre en retour par HCl N/10, en présence de phénolphtaléine.

La méthode employée pour fixer et caractériser l'acétaldéhyde s'inspire des travaux de Neuberg (1919). Elle est décrite par Thomas (1946).

Les autres substances produites par la moisissure sont mises en évidence par chromatographie, en employant les méthodes données dans des notes antérieures (Godin, 1953-1954).

LES RÉSULTATS EXPÉRIMENTAUX.

Influence de l'acide iodoacétique sur le métabolisme.

On a substitué au milieu de culture initial, une solution à 5% de glucose renfermant de l'acide iodoacétique à une des doses croissantes que voici: 0.001 M, 0.0025 M, 0.005 M et 0.0075 M. Les résultats les plus caractéristiques des dosages sont renseignés dans le tableau 1.

La consommation du glucose des solutions substituées renfermant l'acide iodoacétique à une concentration égale ou supérieure à 0.0025 M, ne progresse plus au delà de la valeur indiquée, quelle que soit la durée de l'incubation. Par contre, une concentration en acide iodoacétique égale ou inférieure à 0.001 M n'a pas d'effet sur l'intensité du métabolisme du glucose.

Une autre expérience a été effectuée en substituant au milieu initial, une solution à 3% d'acide gluconique, renfermant de l'acide

TABLEAU 1.

Action de l'acide iodoacétique sur le métabolisme du glucose.

Teneur de la solution substituée en acide iodoacétique	nulle	0.001 M	0.0025 M	0.005 M	0.0075 M
Glucose non consommé, en g, aprés 5 jours d'incubation	2.9	3.2	3.6	3.5	3.65
Id. après 22 jours d'incubation	0.46	0.25	3.65	3.35	3.75

iodoacétique à la concentration 0.01 M. L'acidité titrable de cette solution s'élève, après 5 jours d'incubation, à 174 ml de NaOH N/10 pour 100 ml de solution de métabolisme et se maintient ultérieurement à cette valeur. La consommation de l'acide gluconique est donc bloquée par l'acide iodoacétique 0.01 M.

Les substances formées par la moisissure et décelées par chromatographie de toutes les solutions de glucose renfermant de l'acide iodoacétique, sont l'acide gluconique en faible quantité et le ribose en très faible quantité. En outre, dans les solutions dont la teneur en acide iodoacétique est 0.001 M, on met en évidence les composés phénoliques normalement produits par la moisissure. Dans la solution d'acide gluconique renfermant de l'acide iodoacétique à la concentration 0.01 M, on trouve, comme seule substance produite par la moisissure, le ribose en faible quantité. Il faut toutefois signaler qu'on n'a pas recherché dans les solutions de métabolisme, les polyols éventuellement formés par la moisissure.

Influence du fluorure de sodium sur le métabolisme.

On a substitué au milieu initial, une solution à 5% de glucose, renfermant de fluorure de sodium à une des concentrations que voici: 0.01 M, 0.025 M, 0.0375 M, 0.05 M et 0.075 M. Le tableau 2 renferme les résultats les plus caractéristiques des dosages.

On constate que dans les solutions dont la teneur en fluorure de sodium est égale ou supérieure à 0.025 M, la disparition du glucose est d'abord très lente. Mais après 30 jours d'incubation, le glucose des solutions substituées renfermant du fluorure de sodium

TABLEAU 2.

Action du fluorure de sodium sur le métabolisme du glucose.

Teneur en NaF de la solution substituée	nulle	0.01 M	0.025 M	0.0375 M	0.05 M	0.075 M
Glucose non consommé, en g, après 5 jours d'incubation	2.9	1.45	3.9	4.1	4.0	3.9
Id. après 30 jours d'incubation	0.0	0.0	3.7	3.85	3.8	3.1
Id. après 40 jours d'incubation	0.0	0.0	3.7	2.75	2.25	1.1

à une concentration supérieure à 0.025 M, disparait d'autant plus rapidement que la teneur en fluorure est plus élevée.

Une autre expérience a été réalisée en substituant au milieu initial une solution à 3 % d'acide gluconique et à 0.01 % de $MgHPO_4.3H_2O$, renfermant du fluorure de sodium à une des concentrations suivantes: 0.001 M, 0.00175 M, 0.0025 M et 0.01 M. Les résultats les plus caractéristiques des dosages sont indiqués dans le tableau 3.

TABLEAU 3.

Action de NaF sur le métabolisme de l'acide gluconique.

Teneur en NaF de la solution substituée	nulle	0.001 M	0.00175 M	0.0025 M	0.01 M
Ac.gluconique non consommé, en g, après 5 jours d'incubation	0.5	1.8	2.15	2.4	2.9
Id. après 14 jours d'incubation	0.0	0.0	0.2	1.8	2.65
Id. après 27 jours d'incubation	0.0	0.0	0.0	0.0	0.1

On voit que la consommation de l'acide gluconique est d'autant plus lente que la concentration en fluorure est plus élevée. Dans les conditions réalisées, le fluorure inhibe plus le métabolisme de l'acide gluconique que celui du glucose. Dans le cas où la concentration en fluorure s'élève à 0.01 M, la consommation de l'acide gluconique, de très lente qu'elle était durant les 14 premiers jours de l'incubation, devient brusquement plus rapide durant les deux semaines qui suivent.

Dès avant le quatrième jour de l'incubation, on décèle par chromatographie, dans toutes les solutions glucosées additionnées de fluorure de sodium, des quantités notables d'acide gluconique et de faibles quantités de ribose. Dans les solutions d'acide gluconique, on trouve de très faibles quantités de ribose. Mises à part les substances phénoliques, aucun autre composé produit par la moisissure n'est mis en évidence par chromatographie. Il faut toutefois signaler qu'on n'a pas recherché les polyols éventuellement produits par la moisissure.

Influence des ions cuivriques sur le métabolisme.

Les solutions substituées, à 5% de glucose, renferment des doses croissantes de chlorure cuivrique: 0.001 M, 0.0025 M, 0.005 M, 0.01 M et 0.015 M. Les résultats les plus intéressants des dosages sont donnés dans le tableau 4.

TABLEAU 4.

Influence des ions cuivriques sur le métabolisme du glucose.

Teneur en $CuCl_2$ des solutions substituées	nulle	0.001 M	0.0025 M	0.005 M	0.01 M	0.015 M
Glucose non consommé, en g, après 8 jours d'incubation	1.65	1.8	2.8	3.15	3.1	3.2
Id. après 32 jours d'incubation	0.0	0.0	0.9	2.3	3.0	3.2

On voit que l'addition de chlorure cuivrique à la solution de glucose ralentit d'autant plus la consommation de ce sucre que la teneur en ions Cu est plus grande. Après 67 jours d'incubation, la concentration en glucose des solutions substituées dont la teneur en chlorure cuivrique est égale ou supérieure à 0.01 M, reste identique aux valeurs trouvées précédemment.

Les substances produites par la moisissure aux dépens du glucose et décelées par l'analyse chromatographique de toutes les solutions substituées renfermant du chlorure cuivrique, sont: l'acide gluconique en abondance, le fructose et le mannitol en quantité assez abondante, le ribose et le glycérol en faible quantité. Dans les solutions substituées dont la teneur en chlorure cuivrique ne bloque pas le métabolisme, on décèle aussi les substances phénoliques produites par la moisissure.

Influence de l'azide sodique sur le métabolisme.

La solution substituée renferme 5% de glucose et de l'azide de sodium à une des concentrations que voici: 0.00025 M, 0.0005 M, 0.001 M et 0.002 M. Les résultats les plus caractéristiques des dosages sont donnés dans le tableau 5.

TABLEAU 5.

Influence de l'azide sodique sur le métabolisme du glucose.

Teneur en NaN_3 de la solution substituée	nulle	0.00025 M	0.0005 M	0.001 M	0.002 M
Glucose non consommé, en g, après 10 jours d'incubation	0.71	3.5	3.85	3.85	3.95
Id. après 84 jours d'incubation	0.0	0.05	3.45	3.7	3.45

Une teneur en azide de sodium égale ou supérieure à 0.0005 M bloque donc pratiquement le métabolisme. Une concentration moindre le ralentit considérablement.

La chromatographie de toutes les solutions substituées renfermant de l'azide de sodium permet de déceler les substances suivantes produites par la moisissure aux dépens du glucose: l'acide gluconique en notable quantité, le ribose en faible quantité, le fructose, le mannitol et le glycérol en très faible quantité.

Influence du sulfite de calcium sur le métabolisme.

Au milieu initial, on substitue une solution à 5% de glucose, additionnée d'environ 5 g de sulfite de calcium fraîchement préparé. Dans le tableau 6, on donne le résultat des dosages et des tests effectués sur la solution de métabolisme.

TABLEAU 6.

Influence du sulfite de calcium sur le métabolisme du glucose.

Nature de la solution glucosée substituée	dépourvue de $CaSO_3$	additionnée de $CaSO_3$
Glucose non consommé, en g, après 6 jours d'incubation	2.25	1.35
Id. après 8 jours d'incubation	1.8	0.4
Mise en évidence d'acétaldéhyde dans la solution de métabolisme	—	+

L'acétaldéhyde est mis en évidence par les réactions de Schiff et de Rimini-Lewin, cette dernière étant spécifique. Pour une identification plus certaine, on a distillé la solution de métabolisme sur un excès de $CaCO_3$. Les premières fractions du distillat sont récoltées dans un peu d'eau distillée: elles renferment l'acétaldéhyde. Ce corps est alors précipité sous forme de 2,4-dinitrophénylhydrazone dont le point de fusion obtenu est 145°C. (147°C. dans la littérature).

Le sulfite de calcium active donc la consommation du glucose par la moisissure. Dans les conditions de l'expérience, la fixation de l'acétaldéhyde par $CaSO_3$ n'est que transitoire, car, dès que le glucose est totalement consommé, il n'est plus possible de déceler l'aldéhyde acétique dans la solution de métabolisme.

Par chromatographie de la solution de métabolisme, on met en évidence les substances phénoliques produites en abondance par la moisissure et de très faibles quantités de ribose.

Discussion.

Des inhibiteurs expérimentés, le chlorure cuivrique et l'acide iodoacétique arrêtent définitivement le métabolisme dès que leur concentration est suffisamment élevée. Toutefois, même si la concentration nécessaire pour bloquer le métabolisme est atteinte, une partie du glucose de la solution substituée disparait: 1.42 g par culture si le métabolisme est bloqué par l'acide iodoacétique et 1.85 g si l'inhibiteur utilisé est le chlorure cuivrique.

De ce glucose consommé, une certaine quantité, d'ailleurs minime et, en principe, identique dans les deux cas envisagés ici, diffuse simplement du milieu de culture dans le mycélium. L'autre fraction, la plus importante, a subi des transformations donnant notamment naissance aux produits qui se forment dans les étapes de la dégradation du glucose qui précèdent la réaction empêchée par l'inhibiteur employé. On comprend que cette dernière fraction soit d'autant plus grande que la réaction enzymatique bloquée est plus éloignée du point de départ du processus. Ainsi, en présence d'ions Cu qui empoisonnent la carboxylase, la quantité de glucose consommé est plus grande qu'en présence d'acide iodoacétique qui s'attaque à la phosphoglycéraldéhyde-déshydrogénase.

Les intermédiaires ainsi formés s'accumulent en quantité plus ou moins abondante, dépendant de la valeur de la constante d'équilibre aussi bien de la réaction qui leur donne naissance que de celle qui leur fait subir la transformation ultérieure. Les produits ainsi accumulés dans le mycélium peuvent subir des réactions ,,parasites'' (déphosphorylation, réduction, etc.) et diffuser dans le milieu de culture. Ceci explique comment, malgré un arrêt du métabolisme dû à une concentration suffisamment élevée d'un inhibiteur, certains intermédiaires puissent être mis en évidence par chromatographie du milieu de culture.

En chromatographiant les solutions de glucose additionnées d'acide iodoacétique, de fluorure de sodium, de chlorure cuivrique ou d'azide sodique, on met en évidence, même si les doses de l'inhibiteur employé bloquent le métabolisme, l'acide gluconique et le ribose. Ce dernier corps se trouve également dans la solution d'acide gluconique additionnée d'acide iodoacétique ou de fluorure de sodium. La production de ces substances n'est donc pas empêchée par les inhibiteurs en question, du moins aux doses expérimentées. L'acide gluconique est très vraisemblablement produit par l'action de la notatine ou glucose-aérodéshydrogénase, enzyme très répandu chez les *Aspergillus* et les *Penicillium*. La présence constante de ces deux corps, mise en relation avec la découverte de l'acide 2-cétogluconique parmi les produits du métabolisme du glucose chez *P. brevi-compactum* (Godin, 1953-1954), suggère pour les premières étapes de l'oxydation directe du glucose, le schéma indiqué dans la figure 1. Comme Dickens et Glock (1952) l'ont supposé chez les animaux supérieurs, la décarboxylation de l'acide 2-cétogluconique en ribose serait, ici aussi, précédée d'une énolisation de l'acide, ce qui expliquerait que le pentose formé soit le ribose et non, comme on aurait pu s'y attendre, l'arabinose.

Il est possible que dans ce processus, certaines des substances

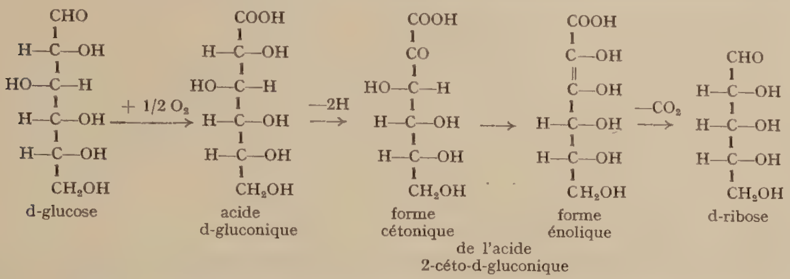

Fig. 1. Schéma proposé des premières étapes de l'oxydation directe du glucose chez *P. brevi-compactum*.

interviennent sous forme d'esters phosphoriques. Dans ce cas, la mise en évidence des corps libres s'expliquerait par l'action d'une phosphatase sur les esters phosphoriques correspondants.

D'autres indications sur l'évolution ultérieure de l'oxydation directe du glucose peuvent être déduites des expériences décrites. Ainsi, le fait que l'acide iodoacétique qui agit sur la phosphoglycéraldéhyde-déshydrogénase, inhibe le métabolisme du glucose tout autant que celui de l'acide gluconique, fait supposer que la dégradation de ce dernier corps aboutit nécessairement au stade de triose-phosphate et qu'à partir de ce stade, le processus se continue comme dans la glycolyse. Le même raisonnement peut être tenu pour le ribose dont l'acide iodoacétique n'empêche pas la production aux dépens de glucose ou d'acide gluconique, mais bien la dégradation ultérieure.

Cette vue est confirmée par les expériences faites avec le fluorure sodique. Non seulement cet inhibiteur de l'énolase influence le métabolisme du glucose aussi bien que celui de l'acide gluconique, mais, après addition de 0.01% de $MgHPO_4.3H_2O$ à la solution d'acide gluconique, la concentration en fluorure nécessaire pour inhiber le métabolisme de cet acide est notoirement inférieure à celle requise pour influencer le métabolisme du glucose en l'absence de phosphate de magnésium. Ceci semble indiquer que l'action inhibitrice du fluorure porte bien sur l'énolase, car, depuis les travaux de WARBURG et CHRISTIAN (1942), on sait que plus la teneur en ions magnésium est élevée, moindre doit être la concentration en ions fluorures requise pour inhiber l'énolase. Toute fois, la reprise ultérieure de la dégradation du glucose ou de l'acide gluconique après une certaine période d'inactivation du métabolisme par le fluorure, fait supposer qu'un système enzymatique adaptatif, non ou moins susceptible au fluorure, s'est entre-temps formé chez la moisissure.

Les ions cuivriques qui agissent sur la carboxylase, bloquent le métabolisme du glucose lorsque leur concentration dans le milieu est égale ou supérieure à 0.01 M, sans toutefois empêcher qu'à cette concentration, une partie du glucose soit oxydée en acide gluconique et en ribose. Comme, dans ces conditions, la consommation du glucose ne progresse pas au delà d'un certain stade, on peut dire que les ions cuivriques bloquent également le métabolisme de l'acide gluconique et celui du ribose. On en déduit que la dégradation de cet acide et celle du pentose passent obligatoirement par l'étape: acide pyruvique $\rightleftarrows$ acétaldéhyde (carboxylase).

Une autre preuve que cette dernière transformation se réalise dans le processus de dégradation du glucose est fournie par la mise en évidence d'acétaldéhyde dans la solution de glucose additionnée de sulfite de calcium.

Il semble donc des plus vraisemblable que l'oxydation directe du glucose aboutisse au stade de triose-phosphate, via le ribose, et que les transformations ultérieures soient identiques à celles de la glycolyse normale. Les recherches relatées dans cette note ne permettent toutefois pas d'établir si le triose est formé par clivage d'une molécule de ribose en un triose et une molécule à deux atomes de carbone, ou si l'oxydation directe se poursuit jusqu'au triose, par passage intermédiaire par le stade C_4.

On a également vu qu'en présence de doses d'ions cuivriques suffisantes pour bloquer le métabolisme du glucose, une fraction notable de ce sucre n'en est pas moins consommée par la moisissure, donnant notamment naissance à du fructose, du mannitol et du glycérol, à côté d'acide gluconique et de ribose. Il semble donc que les premières étapes de la glycolyse, amenant le glucose à l'état de fructose-diphosphate et clivant ce dernier en deux trioses-phosphates, se réalisent également chez *P. brevi-compactum.* Le fructose et le glycérol mis en évidence proviendraient de l'hydrolyse des esters phosphoriques correspondants sous l'action d'une phosphatase.

Quant à l'action inhibitrice de l'azide sodique, elle prouve que le transfert des groupes phosphates riches en énergie est indispensable au déroulement du métabolisme ternaire de la moisissure.

Résumé.

L'étude de l'action inhibitrice de l'acide iodoacétique, du fluorure

de sodium, du chlorure cuivrique, de l'azide sodique et du sulfite de calcium sur le métabolisme du glucose chez *P. brevi-compactum*, semble établir que la dégradation du glucose par oxydation directe aboutit au stade de triose-phosphate, via l'acide gluconique, l'acide 2-cétogluconique et le ribose. A partir du stade triose-phosphate, les transformations paraissent identiques à celles de la glycolyse normale, jusqu'à et y compris le stade d'acétaldéhyde. Il est aussi des plus probable que les premières étapes du processus glycolytique normal, comprenant notamment la formation de fructose-diphosphate et le clivage de ce corps en deux trioses-phosphates, se réalisent chez *P. brevi-compactum*.

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PRODUCTION DE SUBSTANCES PHÉNOLIQUES AUX DÉPENS DE DIVERS COMPOSÉS TERNAIRES PAR *PENICILLIUM BREVI-COMPACTUM*

par

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(Reçu le 26 Mai 1954).

Dans des notes antérieures (GODIN, 1953), on a signalé que *P. brevi-compactum* (souche Db182A) produit les cinq substances phénoliques identifiées par RAISTRICK et coll. On a également démontré que le métabolisme de l'acide mycophénolique est différent de celui des autres substances phénoliques. En effet, ce corps n'est pas synthétisé par la moisissure, après substitution d'une solution de glucose au milieu de culture initial, alors que, dans ces conditions, les autres composés phénoliques sont produits en abondance. Ces derniers, les seuls à nous intéresser ici, semblent, à la suite des travaux d'OXFORD et RAISTRICK (1933), dériver les uns des autres par un jeu d'oxydations ou de réductions successives, de telle sorte que la synthèse, au sens strict du mot, porterait sur une seule des substances de la série, la substance phénolique initiale.

La présente note décrit les premières expériences faites pour élucider le processus de synthèse de la substance phénolique initiale à partir du glucose. Dans ce but, on a recherché quels sont les composés ternaires transformés par la moisissure en substances phénoliques. Les expériences ont porté soit sur des substances que la moisissure produit à partir du glucose et qu'on trouve par analyse chromatographique du milieu de culture (GODIN, 1953) ou du mycélium (GODIN, 1954), soit sur des composés susceptibles d'être des intermédiaires du métabolisme, quoiqu'ils n'aient point été décelés lors des analyses chromatographiques en question.

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MÉTHODES EXPÉRIMENTALES.

Conditions de culture.

La souche de *P. brevi-compactum* utilisée est cataloguée sous l'indicatif Db182A dans la mycothèque de BIOURGE (Laboratoire de Microbiologie de l'Université de Louvain).

Pour étudier l'action de la moisissure sur les différentes substances expérimentées, on emploie la méthode de substitution décrite par FOSTER (1944). La moisissure est ensemencée sur le milieu Czapek-Dox ou le milieu Raulin-Thom. Après développement du mycélium en surface, à 25°C., on remplace aseptiquement le milieu de culture initial par la solution du corps expérimenté.

Dosage des substances phénoliques.

La présence des substances phénoliques dans la solution substituée est décelée par la coloration pourpre, plus ou moins intense, que prend cette solution lorsqu'on y ajoute quelques gouttes d'une solution de $FeCl_3$. Le dosage des substances phénoliques contenues dans la solution de métabolisme est réalisé par photométrie, en mettant à profit cette réaction de coloration. On opère dans le photomètre de Hellige muni du filtre vert et du filtre jaune. Dans les cuves de l'appareil, 1 ou 5 ml de la solution de métabolisme, suivant sa concentration en substances phénoliques, sont portés à 70 ml par addition d'eau distillée. La première cuve sert d'élément de comparaison. On la place dans le faisceau lumineux et amène l'aiguille du voltmètre sur la graduation 100 mV. On ajoute ensuite 5 ml d'une solution à 5% de $FeCl_3$ dans la seconde cuve, mélange le tout et fait glisser la cellule dans le faisceau lumineux. Le nombre de mV indiqué au voltmètre est reporté sur un graphique dont la courbe a été dressée à partir de l'acide phénolique $C_{10}H_{10}O_6$ pur. On en déduit la concentration de la solution expérimentée en acides phénoliques supposés n'être que du $C_{10}H_{10}O_6$.

Comme des composés phénoliques autres que $C_{10}H_{10}O_6$ sont également présents dans la solution, cette méthode de dosage n'est qu'approximative. On doit toutefois noter, d'une part, que $C_{10}H_{10}O_6$ représente toujours, à lui seul, plus de la moitié de la quantité totale des acides phénoliques produits par la moisissure et, d'autre part, que le spectre d'absorption du complexe ferrique de $C_{10}H_{10}O_7$ et celui du complexe ferrique de $C_8H_6O_6$ sont peu différents de celui du complexe de $C_{10}H_{10}O_6$. Quant aux autres substances phénoliques, leur concentration dans la solution de métabolisme est trop faible pour influencer le dosage.

Le dosage ainsi réalisé des substances phénoliques permet de calculer ce qu'on appelle le rendement moléculaire. C'est le rapport du nombre de molécules-grammes des substances phénoliques produites, supposées n'être que du $C_{10}H_{10}O_6$, à la quantité consommée de la source de carbone expérimentée, quantité exprimée elle aussi en molécules-grammes. Ce rapport est calculé lorsque la consommation de la source de carbone étudiée est totale. La disparition de cette dernière est vérifiée soit par polarimétrie (sucres), soit par titrimétrie (acides), soit encore par chromatographie (autres corps).

LES RÉSULTATS EXPÉRIMENTAUX.

Aspect qualitatif de la production des substances phénoliques.

De nombreuses substances ont été expérimentées dans le but de savoir si elles sont consommées par la moisissure et si cette consommation s'accompagne de la production de substances phénoliques. Comme ces expériences ont été faites dans des conditions variables (milieux de culture initiaux différents, volume des milieux, des solutions substituées et des récipients employés différents, etc.), on n'a pu comparer entre eux les résultats quantitatifs obtenus. Il n'en sera donc pas fait mention dans ce paragraphe.

Sont consommés par la moisissure, avec production de quantités plus ou moins abondantes de substances phénoliques, tous les monoses expérimentés: d-glucose, d-mannose, d-galactose, d-sorbose, d-fructose, l-rhamnose, d-arabinose, l-arabinose, d-xylose et d-ribose; les polyalcools que voici: d-sorbitol, d-mannitol, i-inositol, i-érythritol et glycérol; les acides dérivés des sucres ci-après: gluconique, 2-cétogluconique et glucuronique; les acides di- et tricarboxyliques suivants: citrique, itaconique, succinique, malonique, fumarique, malique, tartrique et α-cétoglutarique; les alcools que voici: éthanol et propanol.

Par contre, les acides acétique, glycolique, glycérique, pyruvique et oxalique sont consommés par la moisissure sans donner lieu à la production de substances phénoliques.

Enfin, certains composés ne sont pas dégradés par la moisissure, notamment l'acide isocitrique.

Certaines des substances expérimentées ne sont dégradées par la moisissure que si leur concentration dans la solution substituée est inférieure à la valeur donnée ci-après: acide acétique: 0.5%, acide pyruvique: 0.75% et acide oxalique: 1%. Si leur concentration est plus élevée que la valeur indiquée, ces corps bloquent complètement le métabolisme.

La plupart des acides expérimentés ne sont attaqués par la moisissure que si le pH de la solution substituée est supérieur à 2.5. Comme, dans la plupart des cas, la concentration initiale en acide des solutions substituées s'élève à 3%, on a dû neutraliser partiellement ceux d'entre eux qui, à cette concentration, amènent le pH à une valeur inférieure à 2.5. Cette neutralisation partielle s'effectue en additionnant la quantité voulue de Na_2CO_3 ou, de préférence, de $CaCO_3$, car une concentration trop élevée en ions Na est préjudiciable à la biosynthèse des composés phénoliques.

Il s'avère que la moisissure est capable de métaboliser un grand nombre de substances ternaires: sucres, polyols, alcools et acides les plus divers. Le système enzymatique de la moisissure, qu'il s'agisse d'enzymes constitutifs ou d'enzymes adaptatifs, est donc fort complexe et très ,,malléable''. Ceci ne facilite évidemment pas l'étude de son métabolisme.

On constate aussi que la moisissure est capable de transformer en substances phénoliques des composés ternaires très différents. On peut en déduire qu'il existe une interconnexion entre les divers processus du métabolisme ternaire général de la moisissure et entre ceux-ci et le processus de synthèse des composés phénoliques.

ASPECT QUANTITATIF DE LA PRODUCTION DES SUBSTANCES PHÉNOLIQUES.

Comme la composition du milieu de culture a une influence sur le métabolisme de la moisissure, on a effectué deux séries d'essais: la première en utilisant le milieu Raulin-Thom, la seconde en employant le milieu Czapex-Dox. Dans les deux cas, la moisissure est ensemencée sur 200 ml de milieu de culture, contenus dans des erlenmeyers de 500 ml de capacité. Après 15 jours de développement en surface du mycélium, on substitue au milieu de culture initial, 100 ml de la solution expérimentée.

Cultures développées sur le milieu Raulin-Thom.

Les divers composés expérimentés sont indiqués dans le tableau 1. Tous ces corps sont des produits du métabolisme du glucose chez *P. brevi-compactum* (Oxford et Raistrick, 1933; Godin, 1954). La concentration de chacun d'eux dans la solution substituée est 0.25 M. Après 34 jours d'incubation, on a dosé photométriquement les substances phénoliques produites et on a déterminé le poids sec du mycélium. A ce moment, tous les substrats expérimentés étaient totalement consommés par la moisissure. Les résultats obtenus sont indiqués dans le tableau 1.

TABLEAU 1.

Production de substances phénoliques aux dépens de différentes substances ternaires, par des cultures provenant du milieu Raulin-Thom.

Substances expérimentées	Nombre d'exemplaires	Concentration finale en acides phénoliques	Rendement moléculaire	Poids sec du mycélium par culture, en g
d-glucose	5	90	0.016	3.00
d-fructose	5	83	0.015	2.865
l-arabinose	5	51	0.009	2.80
d-ribose	1	82	0.015	2.80
d-mannitol	5	76	0.0135	3.37
i-érythritol	5	27	0.005	2.80
glycérol	5	57	0.010	2.00
d-gluconolactone	5	120	0.021	2.815
ac. l-malique	3	38	0.007	2.345
ac. succinique	4	36	0.0065	2.505
ac. citrique	5	48	0.0085	2.63

Cultures développées sur le milieu Czapek-Dox.

La composition des solutions substituées et les résultats obtenus sont donnés dans le tableau 2. Lorsque les dosages ont été effectués, les substances expérimentées étaient totalement consommées par la moisissure.

TABLEAU 2.

Production de substances phénoliques aux dépens de différentes substances ternaires, par des cultures provenant du milieu Czapek-Dox.

Substances expérimentées	Concentration initiale en %	Nombre d'exemplaires	Durée de l'incubation en jours	Concentration finale en substances phénoliques	Rendement moléculaire
d-glucose	5	10	25	125	0.0218
ac. d-gluconique	4,5	10	25	165	0.0317
glycérol	5	5	25	68,5	0.0055
ac. succinique	3	5	15	140	0.024
ac. citrique	3	5	15	53	0.0164
ac. d, l-malique	3	5	15	44	0.00875

De l'examen comparatif des tableaux 1 et 2, il ressort que le métabolisme de la moisissure diffère suivant qu'elle s'est développée sur le milieu Czapek-Dox ou le milieu Raulin-Thom. Aussi discutera-t-on séparément les résultats obtenus avec les cultures provenant de chacun de ces milieux.

Cultures provenant du milieu Raulin-Thom.

Des composés ternaires expérimentés, l'acide gluconique est celui qui est transformé par la moisissure en substances phénoliques avec le rendement moléculaire le plus élevé, supérieur même à celui obtenu avec le glucose (0.021 contre 0.016). Par contre, la production des substances phénoliques aux dépens du fructose et du ribose est à peine moindre qu'aux dépens du glucose, tandis qu'à partir du mannitol et surtout de l'arabinose, elle est sensiblement plus petite.

Or, on sait que *P. brevi-compactum* peut oxyder le glucose en acide gluconique et que cette oxydation n'est pas réversible (Nord et Weiss, 1951), mais progresse suivant le processus biochimique appelé oxydation directe du glucose, en donnant notamment naissance au ribose (Godin, 1955). Il est, d'autre part, très vraisemblable que la dégradation du fructose et de son produit de réduction, le mannitol, se réalise principalement par le schéma glycolytique, schéma dont l'existence chez *P. brevi-compactum* semble bien établie (Godin, 1955).

On peut donc déduire de ces propositions et de la comparaison des rendements moléculaires obtenus avec ces diverses substances, d'une part, que le glucose est partiellement métabolisé par la moisissure suivant un processus différent de l'oxydation directe, très vraisemblablement le glycolyse, sinon le rendement moléculaire obtenu avec ce sucre devrait être du même ordre de grandeur que celui obtenu avec l'acide gluconique, et, d'autre part, que le glucose métabolisé par oxydation directe donne lieu à une production de substances phénoliques plus grande que le glucose dégradé par la glycolyse.

Le rendement moléculaire assez élevé obtenu avec le glycérol (0.01) semble cependant infirmer cette dernière hypothèse. En effet, si, comme on l'a supposé, la glycolyse se déroule chez *P. brevicompactum*, on doit s'attendre à ce que le rendement moléculaire obtenu avec le glycérol soit tout au plus égal à la moitié de celui constaté avec le fructose (0.015). Or ce rendement est sensiblement plus élevé que la valeur prévue. Cette anomalie trouve probablement son explication dans le fait que l'assimilation oxydative de la moisissure est beaucoup moins intense quand elle consomme du glycérol que lorsqu'elle consomme du fructose. On voit en effet dans le tableau 1 que, dans le premier cas, le poids sec du mycélium s'élève à 2.00 g par culture, contre 2.865 g pour les cultures qui ont consommé le fructose.

Il semble donc bien établi que la quantité la plus grande de substances phénoliques est obtenue lorsque le glucose est métabolisé par oxydation directe. Ceci peut s'expliquer en admettant qu'un (ou plusieurs) des précurseurs immédiats du composé phénolique initial est produit lors du déroulement de l'oxydation directe du glucose, avec un rendement plus élevé que dans les autres processus du métabolisme ternaire de la moisissure.

Les rendements moléculaires obtenus avec les acides citrique (0.0085), succinique (0.0065) et l-malique (0.007) font supposer que chez la moisissure originaire du milieu Raulin-Thom, ces corps interviennent dans la biosynthèse des substances phénoliques, pour une part minime et proportionnelle au nombre d'atomes de carbone de la molécule.

Quant au i-érythritol, le rendement moléculaire très petit qui y correspond (0.005) fait supposer qu'il n'intervient pas directement dans la production des substances phénoliques.

Cultures provenant du milieu Czapek-Dox.

La biosynthèse des substances phénoliques à partir de l'acide gluconique s'effectue avec un rendement moléculaire notablement plus élevé qu'à partir du glucose (0.0317 contre 0.0218 pour le glucose). D'autre part, le rendement moléculaire obtenu avec le glycérol est particulièrement faible (0.0055). Il semble donc que, chez les cultures originaires du milieu Czapek-Dox également, l'oxydation directe du glucose soit plus efficiente que la glycolyse du point de vue de la production des composés phénoliques.

Les rendements moléculaires obtenus avec les acides di- et tricarboxyliques sont supérieurs à ce qu'on attendrait en se référant aux valeurs obtenues avec les cultures originaires du milieu Raulin-Thom. Le rendement obtenu avec l'acide citrique (0.0164) est environ moitié moindre que celui constaté avec l'acide gluconique (0.0317) et presque le double de celui dû à l'acide d,l-malique (0.00875). Il est possible que seule la forme l- de ce dernier acide, qui est la forme naturelle, intervienne dans le processus de synthèse des substances phénoliques. Quant au rendement moléculaire particulièrement élevé, obtenu avec l'acide succinique (0.024), rendement supérieur même à celui obtenu avec le glucose (0.0218), on peut l'expliquer en admettant que, chez les cultures provenant du milieu Czapek-Dox, ce corps peut intervenir plus directement que les autres acides expérimentés dans le processus de synthèse des substances phénoliques.

D'autre part, la différence constatée entre les rendements moléculaires de l'acide succinique et de l'acide citrique conduit à une supposition importante: chez la moisissure, la dégradation de l'acide citrique ne donnerait pas nécessairement naissance à l'acide succinique et ne s'effectuerait donc pas suivant le processus classique du cycle de KREBS. En effet, en parcourant ce cycle dans son sens normal, une molécule d'acide citrique doit donner naissance à une molécule d'acide succinique. Si tel était le cas chez la moisissure, les rendements moléculaires obtenus avec les acides citrique et succinique devraient être du même ordre de grandeur.

Résumé.

L'étude de la production par *P. brevi-compactum* des substances phénoliques aux dépens de divers composés ternaires, conduit aux principales conclusions que voici:

1° – la moisissure possède un système enzymatique constitutif ou adaptatif fort complexe qui lui permet de dégrader un nombre considérable de substances;
2° – elle est capable de produire ses composés phénoliques aux dépens de corps très différents, ce qui fait présager une interconnexion entre les divers processus de son métabolisme ternaire;
3° – la dégradation du glucose par oxydation directe s'accompagne d'une production de substances phénoliques plus grande que la dégradation de ce même sucre par tout autre processus, notamment la glycolyse;
4° – dans les cultures originaires du milieu Czapek-Dox, il existe, entre le processus de transformation des acides di- et tricarboxyliques et le processus de synthèse des substances phénoliques, une relation plus étroite que chez les cultures provenant du milieu Raulin-Thom, notamment par l'intermédiaire de l'acide succinique;
5° – il semble que le processus de transformation des acides di- et tricarboxyliques chez *P. brevi-compactum* soit différent du cycle de KREBS classique.

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PHAGE REPRODUCTION IN RELATION TO BACTERIAL GROWTH RATE

by

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On infection with a virulent bacteriophage the host bacterium after a "latent period" liberates new phage particles. The latent period can be considered as the time necessary for synthesis and maturation of the phage particles. It is remarkably constant for each virus/host combination even in different media.

Ellis and Delbrück (1939) studied phage propagation at different temperatures and found that the latent period increased with increasing generation times showing that the general slowing down of bacterial metabolism also affected the rate of phage production.

We thought it interesting to know whether, at constant temperature, the latent period also varied with the generation time of the host bacteria.

Among the quantitative methods with which the propagation of a bacteriophage in a bacterial cell can be studied the "one step growth curve" or single cycle experiment takes a prominent place (Ellis and Delbrück, 1939). In this kind of experiment bacteria and phage are mixed in proportions to permit rapid adsorption of the major part of the phage particles. The mixture is then diluted 10 to 10.000 times to prevent readsorption of liberated phage particles. In this diluted culture the number of phage particles is determined at short intervals by plating with sensitive bacteria. In most cases the number of infectious units remains constant during a latent period of 10—40 minutes and then rises steeply to a new constant level. The difference between the two levels represents the "step size" from which the mean "burst size" *i.e.*, the number of phage particles liberated from each host cell can be calculated.

Only bacteria in the logarithmic phase of growth can be used for this kind of experiment. Bacteria from older cultures though they do adsorb phage seem unable to propagate the bacteriophage.

With the usual technic of the one step growth experiment it is not possible however to vary the generation time as one has to use actively multiplying bacteria. It might be possible to use bacteria during the period of slower growth at the end of the log phase but the generation time would be unknown and would change immediately on dilution.

In an apparatus for continuous culture with constant food supply (DE HAAN and WINKLER, 1955) it is possible however to cultivate bacteria at a constant generation time. It seemed possible with such cultures to study the relationship between growth rate of the host bacteria and growth rate (latent period) of the phage.

MATERIALS AND METHODS.

E. coli strain B and phages T_1 to T_7 were used. A simple medium consisting of glucose, ammonium chloride and salts buffered with phosphate was used (WINKLER and DE HAAN, 1948). When necessary dl-tryptophan (30 mg/l) was added. Viable bacteria were counted as usual in our laboratory (JULIUS, 1938). The number of infectious units was estimated by plating with sensitive bacteria (GRATIA 1936, ADAMS, 1950). The continuous culture apparatus has been described (DE HAAN and WINKLER, 1955). The sterilised and assembled apparatus was started at a certain flow rate (generation times of 50′ and 150′) and inoculated with 1 ml of a 24 h culture of *E. coli* B. In about 10 hours a steady state was obtained *i.e.*, the number of bacteria in the vessel was constant and they were growing at a constant generation time. Phage was added after 20 hours and the number of infective units estimated at short intervals.

The number of phage particles in the overflowing liquid was corrected for the dilution factor in the following way: At zero time phage was added and counted after 5 minutes. At the same time the adsorption was determined in the overflowing liquid by killing the bacteria (and the infectious units) with chloroform, which does not harm the free bacteriophage (FREDERICQ, 1950). The number of phage particles was counted at intervals and corrected for the dilution with the formula:

$$\lg N_{t_{corr}} = \lg N_t + \frac{T - 5}{G.T.} \times 0.30103$$

in which formula $\lg N_t$ and $\lg N_{t_{corr}}$ mean the counted resp. the corrected number of phage particles. G.T. means the generation time and $T - 5$ is the time interval between the phage count in question and the count at $T = 5'$ which is taken in all curves as zero level.

In the complete medium the amount of glucose is in excess. This means that in the continuous culture the growth rate of the bacteria is limited not by the energy supply but by some other unknown factor and possibly by the accumulation of toxic products, decrease of pH and so on. With this medium the number of bacteria in the vessel was constant at 4×10^8/ml for a generation time of 50 minutes and at 8×10^8/ml for a generation time of 150 minutes.

By decreasing the amount of glucose to 100 mg/l glucose becomes the limiting factor. For the same generation times of 50′ and 150′ the number of bacteria in the vessel is now of course much lower (about 1.2×10^7/ml). In this latter case the enzymatic apparatus of the bacteria is unharmed by toxic products and only the rate of glucose metabolism determines the growth rate.

Phage propagation was studied under both conditions *i.e.*, in complete medium with glucose in excess as well as in a medium with glucose as a limiting factor.

In preliminary experiments with complete media it seemed that phage adsorbtion was reduced at longer generation times (table 1).

TABLE 1.

The adsorption of phage T_6 in continuous culture at various generation times (the pH was not stabilized).

G.T. in minutes	Adsorption after 5 minutes
51	99%
65	86%
72	71%
90	26%
140	1%

It was established however (table 2) that this effect was due to the decreasing pH of the culture at longer generation times. By changing the ratio of primary and secundary potassium phosphate in the medium (initial pH = 8) this factor could be eliminated.

TABLE 2.

The adsorption of phage T_6 at various pH.

20 ml of an 18 hours culture of *E. coli* B was centrifuged and taken up in 2 ml saline. Immediately afterwards equal parts of this suspension were diluted with 9 volumes of phosphate-buffer of the desired pH. After warming up phage was added. After 5 minutes the adsorption was determined.

pH	phage input/ml	free phage after 5 minutes	phage adsorbed	% adsorption
7.0	5×10^7	0.9×10^7	4.1×10^7	82
6.8	,,	1.9×10^7	3.1×10^7	62
6.5	,,	1.6×10^7	3.4×10^7	68
6.2	,,	2.1×10^7	2.9×10^7	58
5.9	,,	3.9×10^7	1.1×10^7	22
5.5	,,	4.3×10^7	0.7×10^7	14

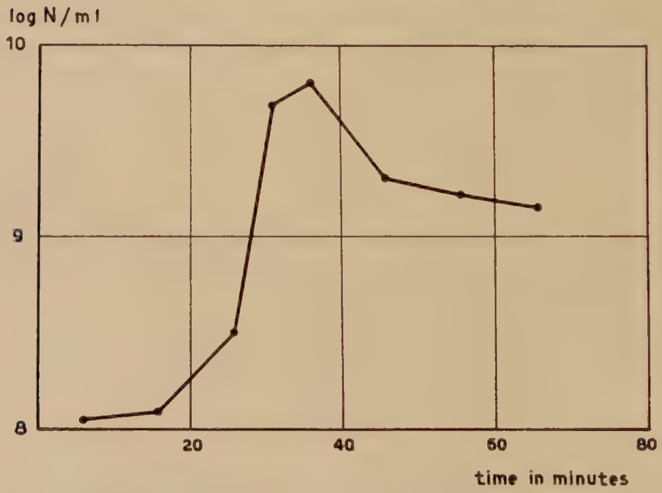

Fig. 1. First step growth curve of phage T_6 in the continuous culture apparatus.

Generation time of *E. coli* B: 65 minutes. Number of bacteria: 7×10^8/ml. Adsorption of bacteriophage after 5 minutes: 91%. Multiplicity of infection after readsorption about 8.

The principle of the one step growth experiment is of course the dilution after adsorption to prevent readsorption. This cannot be realized in a continuous culture as the dilution would disturb the steady state. In preliminary experiments we tried to add so much phage that practically all bacteria would become infected. In these cases the number of infectious units showed a steep rise, as expected but afterwards decreased again presumably by multiple readsorption of phage on the small fraction of bacteria which escaped the first infection (fig. 1).

Reasonable results could be obtained however by adding lower numbers of bacteriophage particles. Such a kind of experiment is not a "single" step growth curve but only a "first" step growth curve. In several of the curves of fig. 3 the beginning of a second reproduction cycle can indeed be seen. Fig. 2 shows that this

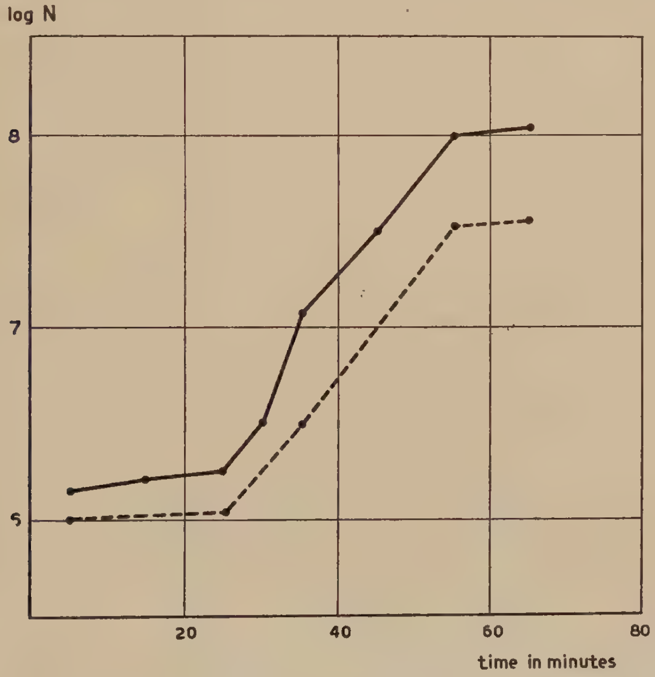

Fig. 2. First step and one step growth curve of phage T_6.

———— First step growth curve of phage T_6 in the continuous culture apparatus. Minimal medium with 30 mg/1 tryptophane. Generation time of bacteria 63 minutes. Number of bacteria 7.5 × 10^8/ml. Phage input 10^6/ml. Adsorption after 5 minutes 60%. The number of phage particles shown is corrected for the continuous dilution of the culture. Step size ± 60.

– – – – – – – One step growth curve of phage T_6. After 5 minutes part of the contents of the continuous culture vessel was taken out and diluted 1 : 100 with minimal medium to prevent readsorption after liberation of phage. Step size under these conditions about 40.

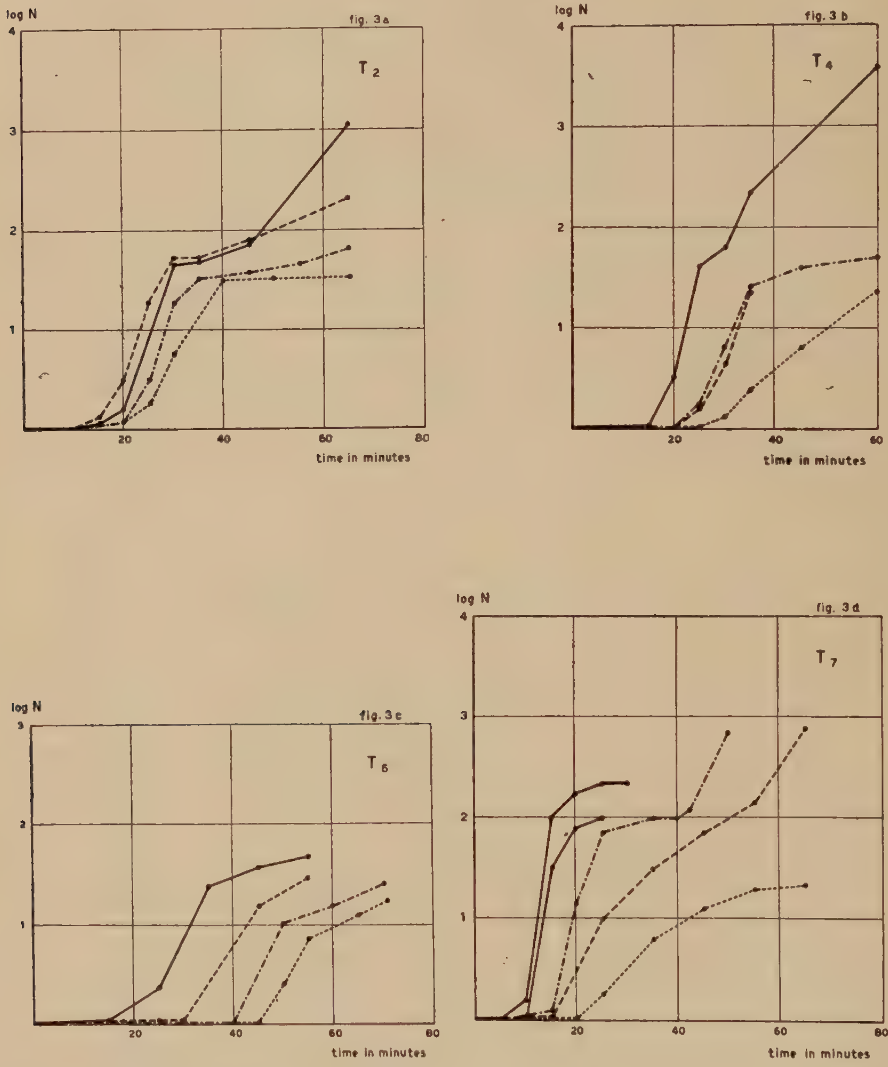

Fig. 3 (*a—d*). First step experiments with phage T_2, T_4, T_6 and T_7 in the continuous culture apparatus.

——————— Generation time 50′ glucose 20 g/l
— — — — — — — — Generation time 50′ glucose 100 mg/l
—.—.—.—.—.—. Generation time 150′ glucose 20 g/l
--------------- Generation time 150′ glucose 100 mg/l

Absis number of phage/ml, corrected for the dilution factor and divided by the number of phage counted after 5 minutes.

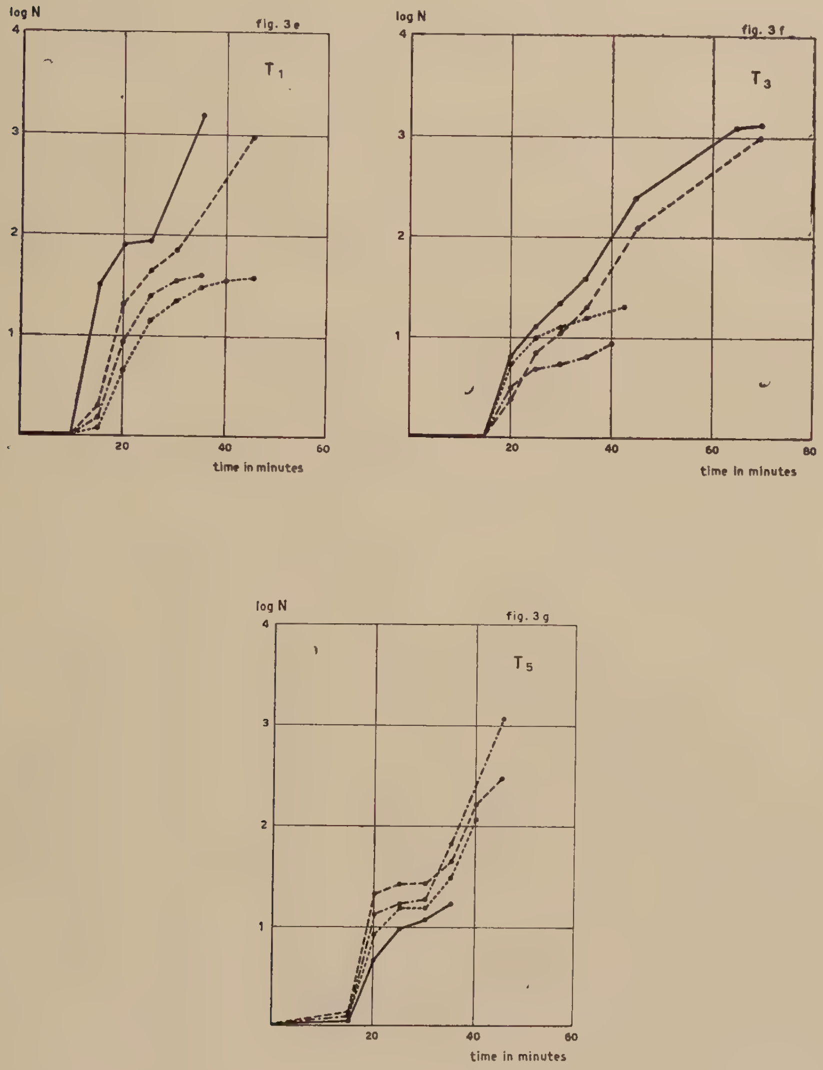

Fig. 3 (*e*—*g*). First step experiments with phage T_1, T_3 and T_5 in the continuous culture apparatus.

——————————— Generation time 50′ glucose 20 g/l
— — — — — — — — — Generation time 50′ glucose 100 mg/l
—.—.—.—.—.—. Generation time 150′ glucose 20 g/l
- - - - - - - - - - - - - - - Generation time 150′ glucose 100 mg/l

Absis number of phage/ml, corrected for the dilution factor and divided by the number of phage counted after 5 minutes.

experimental setup compares well with a conventional one step growth experiment run at the same time and with the same medium parallel with the "first" cycle experiment in the continuous culture apparatus.

RESULTS AND DISCUSSION.

Fig. 3 shows these first cycle experiments of phages $T_1 — T_7$ in complete medium and in a medium with glucose as a limiting factor. Table 3 gives some data about the individual experiments.

TABLE 3.

Approximate latent periods of phages $T_1 — T_7$ at generation times of 50′ and 150′ with high and low supply of glucose.

20 g glucose/l					0.1 g glucose/l		
Phage	G.T.	pH	% ad-sorption	latent period	latent period	% ad-sorption	pH
T_1	50′	6.8	96	±13	14	70	6.7
	150′	6.8	90	14	15	85	—
T_2	50′	6.4	82	18	16	51	6.8
	150′	6.4	74	20	22	79	6.6
T_3	50′	6.3	62	17	18	72	6.7
	150′	6.6	65	18	17	68	6.4
T_4	50′	6.4	90	18	23	60	6.9
	150′	6.2	80	23	28	68	6.9
T_5	50′	6.4	70	16	15	98	6.9
	150′	6.2	75	15	15	83	6.9
T_6	50′	6.3	99	20	30	86	6.6
	150′	6.2	67	40	45	88	6.4
T_7	50′	6.6	80	13	16	97	7.1
	150′	6.7	73	17	22	80	7.1

Increase of bacterial generation time in complete medium results in an increase of the latent period for phages $T_4 — T_6 — T_7$ and to a minor extent for T_2. In some cases a decrease of the slope of the rise is also observed. This means probably that the variation of the individual latent periods of each infected bacterium is larger and can be considered as an increase in mean latent period. The latent periods (and the slopes) of T_1, T_3 and T_5 are much less affected. The burst size — the number of phage particles liberated from each bacterium — is not seriously affected.

In a glucose-limited culture the increase of the bacterial generation time from 50′ to 150′ has about the same effects. Comparison of the curves for the same generation times in the media shows that the reproduction of phages T_2, T_4, T_6 and T_7 is slower in the glucose limited medium than in the medium with glucose in excess.

From these results it is evident that the propagation rate of the bacteriophages T_2, T_4, T_6 and T_7 is not dependent on the generation time of the bacteria per se, but primarily on the energy- and carbon-supply as the longest latent periods were found in the medium with small amounts of glucose.

The bacteriophages T_1, T_3 and T_5 which are synthesized mainly from substances already present in the bacteria and only to a small extent from constituents of the medium (EVANS, 1952) are much less affected by the slowing down of bacterial metabolism and the reduced energy- and carbon-supply.

In a continuous culture apparatus bacteria grow with a definite mean generation time. This does not necessarily mean that every cell divides once during a period of one G.T., but leaves the possibility that a small fraction of the population reproduces at a much higher rate while the other bacteria do not divide though viable on subculture.

The above experiments show that this is not the case but that all bacteria are actively dividing. It is well known that phage reproduction is restricted to actively dividing cells and does not occur in resting cells. If only a fraction of the population were actively dividing this fraction would be smaller the longer the G.T., and as phage reproduction is restricted to actively dividing cells the fraction of bacteria liberating phage would decrease with longer G.T. Such a decrease of the number of infectious units liberating phage would result in a decrease of the step size. (The objection that phage particles were only adsorbed to the fraction of actively dividing bacteria is contrary to experimental evidence).

Fig. 3 shows that the step sizes of the first cycles do not vary with the generation time. We take this as proof that practically the whole viable population is dividing at about the same rate.

Summary.

The reproduction of bacteriophages T_1 — T_7 in *E. coli* B growing in a continuous culture apparatus at constant generation times of 50′ and 150′ was studied in a defined medium with a high con-

centration of glucose and in a medium with glucose as a limiting factor.

It was shown that the propagation rate (as measured by the latent period) of phages T_2, T_4, T_6 and T_7 decreased with longer bacterial generation times and with reduced energy- and carbon-supply. In contrast the reproduction of phages T_1, T_3 and T_5 (which are mainly synthesized from the contents of the host cell) was unaffected by the slowing down of bacterial metabolism.

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BACTERIEN-HAEMAGGLUTINATION

I. VERSUCHE MIT EINEM HAEMAGGLUTINIRENDEN STAMM VON *E. COLI*

von

W. A. COLLIER und **M. JACOEB**

(Eingegangen am 15. Juli 1954).

Einleitung.

Die zuerst durch Hirst (1941) und McClelland und Hare (1941) ausgearbeitete Virus-Haemagglutination stellt nicht nur ein wichtiges Hilfsmittel für die Diagnostik dar, sondern zugleich auch ein Modell für das Studium der Bindung von Zelle und Parasit. In zahlreichen Untersuchungen war man bestrebt, mittels dieser Methodik einen besseren Einblick in den Mechanismus der Pathogenese von Influenza, Pocken, Parotitis epidemica usw. zu gewinnen und hat hierbei ermutigende Resultate erzielt.

Neben den verschiedenen Virusarten, die nur in den Zellen ihres Wirtes zur Entwicklung kommen, kommen auch manche Bacterien intracellulär vor. So finden sich *Myc. leprae* und *Myc. tuberculosis* im Innern bestimmter Zellen und vermehren sich dort. Goodpasture (1937) nimmt an, dass junge Plasmazellen beim Menschen typische Wirtszellen für *E. typhosa* darstellen, die Bacterien ernähren und während der Incubationsperiode und später beschützen. Weiterhin fanden Goodpasture und Anderson (1937), dass epitheliale und mesodermale Zellen der Chorionallantois-Membran als Wirtszellen für *Strept. viridans, A. aerogenes, E. typhosa, Br. abortus* und *Myc. tuberculosis* var. *avium* dienen können. Hierher gehört auch die organotrope Fixierung z.B. der Pneumococcen in der Lunge und die histotrope Fixierung z.B. der Meningococcen in der Hirnhaut. Diese und andere Tatsachen weisen darauf hin, dass zwischen Bacterien und bestimmten Zellreceptoren Affinitäten vorhanden sein müssen, und diese lassen sich möglicherweise mittels der Haemagglutinationstechnik näher analysieren.

Kraus und Ludwig (1902) entdeckten als erste die directe Bacterien-Haemagglutination bei Staphylococcen und Vibrionen. Guyot [1908, cit. nach Kauffmann (1948)] fand später, dass verschiedene Coli-Stämme im Objektträgerversuch Blut agglutinierten. Rosenthal [1943, cit. nach Kauffmann (1948)] erweiterte diese Versuche. Kauffmann (1948) schliesslich untersuchte mittels der Objektträgermethode die Coli-Haemagglutination bei verschiedenen Serotypen und O-Gruppen, und Griffith (1948) bearbeitete die Haemagglutination durch *Sh. alcalescens*, *B. bronchosepticus*, *H. pertussis*, *S. pyogenes* und *V. comma*.

Characterisierung des benutzten Stammes von *E. coli*.

Bei der Untersuchung von haemagglutinierendem Vermögen von Darmbacterien fand sich ein Stamm „65", den Prof. Dr A. Ch. Ruys freundlicherweise für uns determinierte. Nach ihrem Bericht handelte es sich um bewegliche gram-negative Stäbchen mit Kapsel, die auf Bouillonagar glänzende, grauweisse, trübe Kolonien bildeten, auf Endoagar glänzende, rote. Gelatine wurde nicht verflüssigt, aus Nitrat wurde Nitrit gebildet, in Milch Gerinnung und Säurebildung, Wachstum in Koser-Medium negativ, Wachstum in Petruschky-Medium sauer. Indol positiv, Methylrot positiv, Voges-Proskauer negativ, Ureum negativ. In Glucose, Maltose, Saccharose, Lactose, Mannit, Laevulose, Galactose, Trehalose, Xylose, Dulcit, Rhamnose, Raffinose, Arabinose, Mannose Bildung von Säure und Gas, in Glycogen und Salicin weder Säure noch Gas, in Amylum und Inulin Entfärbung und keine Gasbildung.

Allgemeine Technik.

Die Agglutinationsversuche wurden in sterilen Wassermann-Röhrchen aus Pyrexglas durchgeführt. Falls nicht anders angegeben, wurde Hühnerblut gebraucht, das in Natriumcitrat aufgefangen und dreimal mit physiologischer Kochsalzlösung gewaschen wurde. Die abcentrifugierten Erythrocyten wurden im Eisschrank aufbewahrt und direct vor Gebrauch in Kochsalzlösung suspendiert. Das Alter des Blutes (1 bis 4 Tage) schien keinen Einfluss auszuüben.

Alle Lösungen, Suspensionen und Verdünnungen wurden in physiologischer Kochsalzlösung hergestellt.

Technik des Haemagglutinationsversuches.

In einer Reihe von Röhrchen wurden fallende Verdünnungen der Bacteriencultur in Zweierpotenzen in einem Volumen von 0,5 ml hergestellt. Hierzu wurde je 0,5 ml einer 1%igen Suspension von Hühnererythrocyten gebraucht. Nach Durchschütteln blieben die Röhrchen bei Zimmertemperatur (22—25°C.) stehen. Abgelesen wurde nach völliger Sedimentierung der Erythrocyten nach etwa 1½—2 Stunden. Das Ergebnis wurde als stark positiv (+++) bezeichnet, wenn die Erythrocyten die Kuppe des Röhrchens als dicker Film bedeckten, als negativ (o), wenn auf dem Boden ein roter Knopf mit scharfem Rand zu sehen war. Als (+) wurde das Ergebnis bezeichnet, wenn um die Knopfbildung am Boden mit dem blossen Auge noch deutlich einzelne Häufchen agglutinierter Erythrocyten zu erkennen waren.

Die Berechnung der Verdünnungen erfolgte stets für die Endconcentration im Hinblick auf das Gesamtvolumen. Waren also im ersten Röhrchen 0,5 ml unverdünnte Cultur und 0,5 ml Blutsuspension, so wurde als Bacterienverdünnung 1/2 angegeben.

Culturmedium.

Die Bacterien wurden ausnahmslos in Difco-Bouillon bei 37° gezüchtet, häufig aber nach etwa 2 Wochen bei Zimmertemperatur aufbewahrt.

Haemagglutination verschiedener Blutarten.

Es standen uns nicht viel verschiedene Blutarten zur Verfügung. Eine Uebersicht findet sich in Tabelle 1.

TABELLE 1.
Haemagglutination des Blutes verschiedener Tierarten durch *E. coli*, Stamm 65.

Blut von:	Titer:
Huhn (± 100)	1/32—1/2048
Ente	1/8—1/32
Maus	1/64
Meerschwein	1/256—1/1024
Stachelschwein	1/2048
Rind	0—1/64
Schwein	1/512
Schaf	0—1/128
Mensch (39) (O, A, B, AB)	0—1/512

Von mehr als 100 Hühnern zeigte das Blut eines Tieres bei Zimmertemperatur in Kochsalzlösung Spontanagglutination. Sonst war das Blut jedes Huhnes von jedem Alter brauchbar, wenn sich auch Differenzen in der Agglutinierbarkeit zeigten.

Bei der Untersuchung verschiedener Blutmuster gegenüber der gleichen Cultur ergaben in einem Versuch vom 5 Mustern 2 einen Titer von 1/32 und 3 einen solchen von 1/64. Bei einer weiteren Probe von 7 Blutmustern wurde einmal 1/64, dreimal 1/128, zweimal 1/256 und einmal 1/512 gefunden. In einem 3 Versuch ergab sich dreimal ein Titer von 1/640, sechsmal von 1/1280 und einmal ein noch höherer Titer.

Vom Menschen wurden 39 Blutproben untersucht. Hier war nicht jedes Blut agglutinierbar, die Titer schwankten zwischen 0—1/512. Ein Zusammenhang mit dem O-A-B-System bestand nicht, da in jeder Gruppe sowohl empfindliches als auch nicht-reagierendes Blut vorkam.

Stabilität der Bindung.

Die Bindung zwischen Haemagglutinin und Hühnererythrocyten ist relativ fest. In einigen Fällen gelang es nicht, durch einfaches Schütteln der ersten Röhrchen einer Verdünnungsreihe die Erythrocyten wieder völlig voneinander zu trennen. Es blieben feine, mit dem blossen Auge eben noch erkennbare Körnchen zurück. Nach dem Aufschütteln kam die Haemagglutination stets wieder zurück. Die Receptoren blieben also wirksam.

Dies ergab sich deutlich bei einem Versuch mit 3 Verdünnungsreihen. Die 1. und 2. Reihe wurde bei 37° bzw. bei Zimmertemperatur beobachtet und nach 2, $4\frac{1}{2}$, $5\frac{1}{2}$, 9, 24 und 28 Stunden wieder aufgeschüttelt. Die 3. Reihe wurde bei 4° gehalten und nach 3, $4\frac{1}{2}$, 9, 24 und 28 Stunden aufgeschüttelt. In allen drei Fällen wurde jedesmal nach dem Aufschütteln wiederum Haemagglutination in der Höhe des erstmal festgestellten Titers erhalten.

Haemagglutination durch fractionierte Cultur.

Nicht nur die Vollcultur der Bacterien ist imstande, Hühnererythrocyten zu agglutinieren, sondern auch die verschiedenen Fractionen. Von zahlreichen Versuchen sind zwei in der Tabelle 2 wiedergegeben.

TABELLE 2.

Haemagglutination von Hühnerblut durch Cultur-Fractionen von *E. coli*, Stamm 65, bei verschiedenen Temperaturen.

Haemagglutination durch:	Versuch 1	Versuch 2		
	± 28°	± 4°	± 28°	37°
Vollcultur	1/256	1/512	1/512	1/512
bacterienfreies Medium	1/128	1/128	1/256	1/256
abcentrifugierte Bacterien	1/16			
ausgewaschene Bacterien		1/32	1/32	1/16
1. Waschwasser hiervon		1/16	1/32	1/16

Im ersten Versuch wurden die Bacterien abcentrifugiert und in der gleichen Menge physiologischer Kochsalzlösung resuspendiert. Die Vollcultur ergab einen Titer von 1/256, das Medium allein 1/128, und die resuspendierten Bacterien 1/16.

Im andern Versuch wurden die abcentrifugierten Bacterien mit Kochsalzlösung gewaschen und hiernach resuspendiert. Die verschiedenen Fractionen wurden bei 4°, 28° und 37° untersucht. In allen drei Fällen ergab die Vollcultur einen Titer von 1/512, das Medium allein Titer von 1/128—1/256, die gewaschenen und resuspendierten Bacterien und ebenso das Waschwasser Titer von 1/16—1/32. Wurden Bacterien mehrmals gewaschen, so agglutinierte nur das erste Waschwasser, nicht aber die späteren.

Einfluss der Temperatur auf die Haemagglutinine.

Die Haemagglutinine erwiesen sich einer Temperatur von 56° gegenüber in der Regel als resistent, während sie durch 63° relativ schnell zerstört wurden. Als Beispiel sei in Tabelle 3 ein derartiger Versuch wiedergegeben.

Von einer gut gewachsenen Cultur wurden 3 Röhrchen des nativen Materials und 3 Röhrchen des völlig bacterienfrei centrifugierten Mediums in je ein Wasserbad von 56°, 63° und 70° gebracht. Nach 10′, 20′, 30′, 60′ und 120′ wurde Material daraus entnommen und zusammen mit der nativen Cultur auf Haemagglutinine untersucht. Die Temperatur von 56° erwies sich als indifferent, denn

TABELLE 3.

Einfluss des Erwärmens auf die Hühnerblut haemagglutinierende Fähigkeit der Vollcultur und des bacterienfreien Mediums von *E. coli*, Stamm 65.

Dauer der Erwärmung	Endtiter der Vollcultur nach Erwärmen				Endtiter des Mediums nach Erwärmen			
	nativ	56°	63°	70°	nativ	65°	63°	70°
—	1/1024				1/256			
10′		1/1024	1/256	>1/2		1/64	>1/2	>1/2
20′		1/1024	1/32	>1/2		1/64	>1/2	>1/2
30′		1/1024	>1/2	>1/2		1/128	>1/2	>1/2
60′		1/1024	>1/2	>1/2		1/256		
120′		1/1024	>1/2	>1/2		1/64		

die erwärmten Bacterien zeigten stets den gleichen Titer wie die native Controlle. Auch im bacterienfreien Medium war nur eine geringe Abschwächung gegenüber dem nativen Medium festzustellen. Anders war es bei der Temperatur von 63°. Die Haemagglutinine in der Cultur waren nach 10′ bereits leicht abgeschwächt, nach 20′ schon stärker, und nach 30′ war jede haemagglutinierende Fähigkeit verschwunden. Das bacterienfreie Medium war bereits nach 10′ wirkungslos. Bei 70° war in jedem Fall nach 10′ keine Haemagglutination mehr feststellbar.

Nur in einigen Ausnahmefällen wirkte bereits die Temperatur von 56° zerstörend auf das Haemagglutinin.

Nicht selten zeigte sich nach 30′ Erwärmen auf 56° im ersten und zweiten Röhrchen einer Verdünnungsreihe keine Agglutination der Hühnererythrocyten, wohl aber sehr stark in den folgenden Verdünnungen.

Haltbarkeit der Haemagglutinine in Bouillonculturen.

In der Tabelle 4 ist in zwei Fällen der Titerverlauf der Haemagglutinine 2 Monate lang verfolgt. Zwei Bouillon-Kolben mit je 200 ml Medium wurden gleichstark beimpft und 15 Tage lang bei 37° und hiernach bei Zimmertemperatur belassen. Zu verschiedenen Zeiten wurde die Haemagglutinationsfähigkeit des nativen und des 30′ lang auf 56° erwärmten Materials bestimmt. Es ergab sich, dass in dem einen Kolben die Titer auch nach 2 Monaten in ungefähr der gleichen Höhe geblieben waren wie nach 2 Tagen. Im andern

Kolben war nach 30 Tagen der Titer des erwärmten Materials sehr niedrig, nach 60 Tagen auch der Titer der nativen Cultur.

TABELLE 4.

Verlauf der Haemagglutinationstiter gegenüber Hühnerblut von zwei Culturen von *E. coli*, Stamm 65, während zweier Monate.

Alter der Culturen	Cultur 1		Cultur 2	
	nativ	30′ 56°	nativ	30′ 56°
2 Tage	1/256	1/64	1/256	1/128
5 Tage	1/512	1/256	1/512	1/128
8 Tage	1/1024	1/256	1/256	1/512
15 Tage	1/512	1/128	1/256	1/512
21 Tage	1/1024	1/512	1/512	1/256
30 Tage	1/512	1/128	1/256	1/4
60 Tage	1/512	1/512	1/16	1/8

Zahlreiche weitere Versuche verliefen analog. So zeigte eine etwa 2 Monate lang stark agglutinierende Cultur nach 77 Tagen nur noch einen Titer von 1/16 und nach 91 Tagen einen solchen von 1/2. Eine andere Cultur zeigte am 46. Tage einen Titer von 1/1024, am 60. Tage einen solchen von 1/64 und am 97. Tage einen Titer von 1/128. Zwei stets bei 37° gehaltene Culturen agglutinierten nach 3 Monaten beide in einer Verdünnung von 1/512, in einem Fall war eine bei Zimmertemperatur gehaltenen Cultur noch nach 8 Monaten stark wirksam.

Zusammenfassend lässt sich feststellen, dass die Haemagglutinine in den meisten Fällen sehr lange feststellbar sind, dass aber mitunter auch nach einiger Zeit ein Titerverlust eintreten kann.

Einfluss der pH auf die Haemagglutination.

Der Einfluss der pH wurde auf die native und 30′ auf 56° erwärmte Cultur sowie auf das bacterienfreie Medium untersucht. Sowohl die fallenden Verdünnungen als auch die Erythrocytensuspension wurde mit Phosphatpuffer-Kochsalzlösung hergestellt. Die Ergebnisse finden sich in Tabelle 5. Bei pH 5,3 kam es zu unspecifischer Haemagglutination und leichter Braunfärbung der Erythrocyten, bei pH 5,9—8,2 aber stimmten die Titer der nativen Cultur mit denen der Kochsalz-Controlle überein. Bei der erwärmten Cultur und dem bacterienfreien Medium war bei pH 8,2 vielleicht eine geringe Titerverminderung festzustellen, die sich aber in anderen Versuchsreihen nicht regelmässig zeigte.

TABELLE 5.
Haemagglutination von Hühnerblut durch *E. coli*, Stamm 65, in m/15 Phosphatpuffer.

pH	Haemagglutinations-Titer:		
	nativ	56° 30′	Medium
5,3	unspecifisch	unspecifisch	unspecifisch
5,9	1/1280	1/640	1/160
7,0	1/1280	1/640	1/160
8,2	1/640	1/320	1/40
NaCl Controlle	1/1280	1/1280	1/160

Einfluss von Formalin.

Wurden die Hühnererythrocyten statt in physiologischer Kochsalzlösung in 0,5%iger Formalin-Kochsalzlösung suspendiert, so verlief die Haemagglutination völlig in Uebereinstimmung mit den Kochsalzcontrollen.

Demgegenüber beeinflusste Formalin die Haemagglutinine in den Culturen. Von 3 verschieden alten Culturen wurden jeweils mehrere Röhrchen mit fallenden Mengen Formalin gemengt und bei 37° bewahrt. Nach 3 Tagen hatte 0,5% Formalin jegliche haemagglutinierende Fähigkeit zerstört, in zwei Fällen auch 0,25% Formalin vollständig und in einem Fall fast völlig. Formalin zerstört also die Haemagglutinine in der Bacteriencultur.

Haemagglutination in Gelatinelösung.

Wurde als Verdünnungsmedium anstelle von Kochsalzlösung Gelatinelösung (in Kochsalzlösung) benutzt, so wurde ein weniger hoher Agglutinationstiter erreicht. Während in einem Versuch beispielsweise in Kochsalzlösung ein Titer von 1/800 gefunden wurde, war der Endtiter bei Verwendung von 0,31% und 0,63% Gelatine-Endconcentration nur 1/200, bei 1,25% Gelatine sogar nur 1/100. Bei Verwendung höherer Gelatineconcentrationen kam es teilweise zu Hämolyse der Hühner-erythrocyten.

Einfluss von Kohlehydraten auf die Haemagglutininbildung.

In einer Versuchsserie wurde je 10 ml Bouillon mit 1% verschiedener Kohlehydrate versetzt, stark beimpft und nach 24 und

48 Stunden auf Haemagglutinine untersucht. Aus Tabelle 6 ergibt sich, dass nach Zusatz von Dextrin, Erythrit, Isodulcit, Mannit, Raffinose, Saccharose und Sorbit ungefähr die gleichen Titer erhalten werden, wie in der kohlehydratfreien Controlle. In der Lactose-Bouillon war in diesem Versuch nach 48 Stunden ein Verlust an Haemagglutininen zu sehen, der in anderen Versuchen nicht festgestellt werden konnte. Ebenso zeigte sich in der Glucose-Bouillon ein starker Titerabfall, doch erwies sich das Medium als stark gesäuert. Völlig abweichend reagierte in diesem Versuch die Mannose-Bouillon, indem sich hier bis 1/32 bzw. 1/16 Hämolyse zeigte; doch auch hier erwies sich das Medium als stark angesäuert. In Bouillon mit 0,5 % Mannose kam es dagegen stets zu einer normal verlaufenden Haemagglutininbildung. Die Zugabe von verschiedenen Kohlehydraten zum Medium scheint also in der Regel keinen Einfluss auf die Ausbildung von Haemagglutininen auszuüben.

TABELLE 6.

Haemagglutination von Hühnerblut durch *E. coli*, Stamm 65, in 1%igen Zuckermedien nach 24 und 48 Stunden.

Nährboden mit 1% Zucker:	Titer nach:	
	24 Stunden	48 Stunden
Dextrin	1/512	1/1024
Erythrit	1/512	1/512
Glucose	1/256	1/16 1)
"Isodulcit"	1/1024	1/1024
Lactose	1/1024	1/128
Mannit	1/1024	1/2048
Mannose	1/32 2)	1/16 1) 2)
Raffinose	1/1024	1/1024
Saccharose	1/1024	1/1024
Sorbit	1/1024	1/2048
Bouillon-Controlle	1/1024	1/512

1) stark sauer! 2) Haemolyse

Adsorption der Haemagglutinine durch Blut oder Bacterien.

In zwei analogen Versuchen wurden von je einer Cultur die Bacterien abcentrifugiert und nur das Medium verwendet. Zur Adsorption wurden native und 30′ lang auf 56° erwärmte Hühner-Erythrocyten benutzt und zwar 0,2 ml zu 2,0 ml Medium. Weiterhin wurden von je 20 ml Cultur von 2 Stämmen *E. coli* die lebenden und von den Stämmen Typhus 901 O, Typhus 901 OH, Proteus O 19 und Proteus OXK die abgetöteten Bacterien abcentrifugiert. Diese Sedimente wurden mit je 5,0 ml der oben erwähnten bacterienfreien Medien gemengt und 30′ bei 37° gebunden. Das Blut bzw. die Bacterien wurden hierauf abcentrifugiert und die überstehende Flüssigkeit und die unbehandelten Medien auf ihre Haemagglutinationsfähigkeit untersucht.

In den beiden Versuchen betrug der Haemagglutinationstiter der unbehandelten Bouillon 1/128 bzw. 1/64. Das mit nativem und erwärmtem Hühnerblut behandelte Medium hatte jegliches Haemagglutinationsvermögen verloren, ebenso die mit den Typhusstämmen, den Proteusstämmen und dem einen Escherichiastamm behandelten Media. Die mit dem zweiten Escherichiastamm behandelten Media wiesen noch Titer von 1/64 bzw. 1/32 auf.

Es zeigte sich also, dass die in dem Culturmedium vorhandenen Haemagglutinine nicht nur specifisch durch natives Hühnerblut, sondern auch durch auf 56° erwärmtes Blut und durch verschiedene Bacterienarten adsorbiert werden können.

Besprechung der Ergebnisse.

Das untersuchte Agglutinin scheint sich nicht nur an den Bacterien, sondern auch im Medium zu befinden, da die Bacterien allein in der Regel schwächer wirken als die Vollcultur oder das Medium selbst. Dies lässt daran denken, dass das wirksame Agglutinin besonders — aber wohl nicht ausschliesslich — in der Kapselsubstanz zu suchen ist.

Die Temperaturempfindlichkeit ist relativ hoch, denn 63° wirken stets abschwächend, in einigen Fällen sogar schon 56°.

Während in der Regel die Haemagglutinine sehr lange (bis zu 8 Monaten) nachweisbar sind, kommt es in einigen Fällen zu einer deutlichen Abnahme innerhalb von 2 Monaten. Da es sich stets um das gleiche Standard-Milieu handelt, ist dies abweichende Verhalten nicht zu erklären.

Während es für den Haemagglutinationsversuch völlig gleichgültig ist, ob die Erythrocyten in Kochsalzlösung oder in 0,5% Formol-Kochsalzlösung suspendiert sind, ist es doch deutlich, dass

Formol zerstörend auf die Haemagglutinine wirkt, wenn es einige Tage auf die Cultur einwirkt.

Eigenartig ist es, dass stärkere Gelatinelösung nicht nur die Reaction verlangsamt, was ja zu erwarten ist, sondern sogar den Titer der Agglutinine erniedrigt. Anscheinend ist die Bindung des Agglutinins an die Erythrocyten bei dieser Versuchsanordnung nicht complett.

Zusammenfassung.

Der mit sehr starker Kapselbildung wachsende Stamm „65" von *E. coli* agglutinierte stark verschiedene Blutarten. Von Hühnern war jedes Blut geeignet, vom Menschen dagegen nicht. Die Bindung ist relativ fest, die Receptoren werden nicht zerstört. Das von Bacterien frei centrifugierte Medium enthält die Mehrheit der Agglutinine. Eine Temperatur von 63° wirkt zerstörend auf die Haemagglutinine. In Bouillonculturen sind die Haemagglutinine bis zu 8 Monaten nachweisbar. Die Reaction verläuft innerhalb pH 5,9—8,2 völlig unbeeinflusst. Formalin zerstört innerhalb von 3 Tagen die Haemagglutinine. Zufügung von Kohlehydraten zum Medium ist in der Regel ohne Einfluss auf die Bildung der Haemagglutinine. Diese werden durch Hühnererythrocyten und durch verschiedene Bacterien adsorbiert.

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BACTERIEN-HAEMAGGLUTINATION

II. UNSPECIFISCHE HEMMUNG DER COLI-HAEMAGGLUTINATION [1]

von

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(Eingegangen am 15. Juli 1954).

In der vorangehenden Arbeit haben COLLIER und JACOEB über die Haemagglutination durch einen Stamm „65" von *E. coli* berichtet. Es war nunmehr von Interesse zu untersuchen, ob diese Bacterien-Haemagglutination durch einige Stoffe gehemmt werden konnte, die auch bei der Virus-Haemagglutination diese Eigenschaft zeigten.

HIRST (1942) wies als erster darauf hin, dass menschliches Normalserum eine unspecifische Hemmung der Virus-Haemagglutination verursachen kann. Diese Erscheinung wurde durch BEVERIDGE und LIND (1946), FRANCIS, SALK und BRACE (1946), BURNET und McCREA (1947), BURNET, McCREA und ANDERSON (1947), FRANCIS (1947), HIRST, VILCHES, ROGERS und ROBBINS (1947), McCREA (1947) u.a. weiterhin untersucht. Ueber die Hemmungswirkung von Erythrocyten- und Gewebsextracten berichteten später BOVARNICK und DE BURGH (1947), FRIEDEWALD, MILLER und WHATLEY (1947), GREEN und WOOLEY (1947) u.a. Ueber die unspecifische Hemmung durch Allantoisflüssigkeit berichtete SVEDMYR (1946), durch Hühner-Eiklaar LANNI und BEARD (1948) und durch Urin TAMM und HORSFALL (1950).

Untersuchungen über die unspecifischen Hemmungsstoffe sind geeignet, nähere Einsichten über die Aviditäten von Zellreceptoren zu ermöglichen, auch bestimmte chemotherapeutische Probleme können dadurch gefördert werden. U.a. könnte auch die Phagocytose ohne Beteiligung von Antistoffen, die WOOD (1947) und KNISLEY, BLOCH und WARNER (1948) ausführlich beleuchten, mit der Frage der Bindung von Zellreceptoren mit nicht-specifischen Eiweissverbindungen zusammenhängen.

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[1] Teil I: Antonie van Leeuwenhoek 21, 113, 1955.

Technik des Hemmungsversuches.

Während im Allgemeinen der durch Collier und Jacoeb (1955) angegebenen Technik gefolgt wurde, ergaben sich bei der Untersuchung der Hemmungswirkung folgende Besonderheiten:

Von der Bacteriencultur wurde die einfache haemagglutinierende Dosis festgestellt. Von der zu untersuchenden Substanz wurden fallende Verdünnungen in Zweierpotenzen hergestellt und zwar in einem Volumen von 0,25 ml. Sollte nun die Hemmungswirkung auf das 4-fache Multiplum der einfach haemagglutinierenden Dosis festgestellt werden, so wurde zu jedem Röhrchen je 0,25 ml des 16-fachen Multiplums der einfach haemagglutinierenden Dosis zugefügt. Nach gutem Durchschütteln wurde im Wasserbad oder Brutschrank von 37°C. oder aber bei Zimmertemperatur gebunden. Die Bindungszeit betrug in der Regel 30 Minuten. Hiernach kam zu jedem Röhrchen 0,5 ml einer 1%igen Hühnererythrocyten-Suspension, und nach 1½—2 Stunden Stehen bei Zimmertemperatur wurde abgelesen.

Die Berechnung der Verdünnungen usw. erfolgte stets im Hinblick auf das Gesamtvolumen. Wurden beispielsweise 0,25 ml einer Normalserumverdünnung von 1/5, 0,25 ml einer Bacteriensuspension von 1/20 und 0,5 ml Blutsuspension gemengt, so wurde die Serumverdünnung 1/20, und die Verdünnung der Bacteriensuspension 1/80 genannt.

Hemmung durch einige Chemicalien.

Durch Methylalcohol, Aethylalcohol, Formalin, Glycerin, Glycol, Acidum tartaricum, Acidum lacticum, Acidum oxalicum, Natriumchlorid, Kaliumchlorid, Magnesiumchlorid, Calciumchlorid, Sublimat und Gummi arabicum wurde in Concentrationen, die auf Hühnerblut keine toxische Einwirkung hatten, keine Hemmung der Haemagglutination beobachtet.

Hemmung durch Urin.

Bei der Untersuchung verschiedener menschlicher Urine zeigte es sich, dass sehr häufig eine mehr oder weniger starke Hemmung der Haemagglutination zu verzeichnen war, gleichgültig ob in den

Urinen mittels der gebräuchlichen Laboratoriumsmethoden Eiweiss nachzuweisen war oder nicht. Saure Urine wurden vor Anstellen des Hemmungsversuches neutralisiert.

Hemmung durch Normalsera.

Es ergab sich, dass normale Hühnersera imstande waren, die Haemagglutination zu hemmen. Sowohl das Serum des gleichen Huhns, von dem die Erythrocyten stammten, als auch die Sera anderer Tiere hemmten deutlich die Reaction. In der Regel ergab sich complette Hemmung bis zu einer Serum-Endverdünnung von 1/80, doch wurden auch höhere Werte erreicht.

Die gleiche Hemmungswirkung zeigte sich auch bei normalem Menschenserum. In der Tabelle 1 sind die Endverdünnungen von 49 inactivierten Seren zusammengestellt, wodurch die 4-fach agglutinierende Dosis einer Bacteriencultur gehemmt wurde. Auch beim Menschenserum lag der Hemmungstiter meistenteils bei einer Endverdünnung von 1/80, doch kamen auch höhere und niedrigere Werte vor. Interessant ist vielleicht, dass 2 von 7 Seren mit einem Titer von 1/40 und das einzige Serum mit einem Hemmungstiter von 1/20 von leberkranken Patienten stammte.

TABELLE 1.

Hemmung der Haemagglutination von Hühnerblut durch *E. coli*, Stamm 65, durch 49 inactivierte Menschensera.

Menschensera	Endtiter der Hemmung
1 Serum	1/320
2 Sera	1/160
38 Sera	1/80
7 Sera	1/40 [1]
1 Serum	1/20 [2]

[1] hierbei 2 Sera von Leberpatienten

[2] Serum eines Leberpatienten.

Eigenartig war das Verhalten der Normalsera beim Erwärmen. In der Tabelle 2 ist die Beeinflussung der Hemmungswirkung von 2 Menschenseren und einem Hühnerserum durch Erwärmen auf 70°, 80° und 100° zusammengestellt.

Im Menschenserum 1, das an sich schon stark hemmend wirkte,

TABELLE 2.

Einfluss des Erwärmens auf die Hemmung der Haemagglutination von Hühnerblut durch *E. coli*, Stamm 65, durch Menschenserum und Hühnerserum.

Temperatuur und Zeit:	Endtiter der Hemmung: Menschenserum 1	Menschenserum 2	Hühnerserum
nativ	1/320	1/40	1/320
70° 5′	1/160	1/160	
10′	1/160	1/160	
20′	1/160	1/320	1/2560
30′	1/1280	1/40	1/1280
60′	1/1280	1/40	1/2560
120′	1/2560	1/40	1/2560
180′		1/320	
80° 5′	1/2560	1/20	1/640
10′	1/1280	1/20	1/2560
20′	1/10240	1/80	1/10240
30′	1/2560	1/80	1/10240
60′	> 1/20	1/640	1/10240
120′	> 1/20	1/1280	1/10240
100° 5′	1/5120	1/320	1/10240
10′	1/2560	1/320	1/10240
15′	> 1/20	> 1/20	
20′	> 1/20	> 1/20	> 1/20

trat bei Erwärmen auf 70° nach 30′ eine Zunahme der Hemmungswirkung auf. Beim Erwärmen auf 80° war bereits nach 5′ eine starke Hemmungssteigerung zu sehen, aber nach 60′ war jegliche Hemmung verloren gegangen. Bei Einwirkung von 100° war nach 5′ und 10′ eine starke Hemmungssteigerung zu erkennen, aber nach 15′ war die Wirkung verschwunden. Etwas anders war der Verlauf bei dem schwach hemmenden Menschenserum 2. Hier war beim Erwärmen auf 70° schon nach 5′ eine Hemmungssteigerung zu verzeichnen, bei dem nach 30′ und 60′ entnommenen Material eigentümlicherweise aber nicht, wohl aber wieder nach 120′ und 180′. Beim Erwärmen auf 80° begann hier die Wirkungssteigerung nach 20′, um nach 120′ ein Maximum erreicht zu haben. Beim Erwärmen auf 100° war nach anfänglicher Hemmungssteigerung nach 15′ wieder jeder Einfluss verschwunden. Bei dem Hühnerserum wurden gleichfalls überall starke Steigerungen des Hemmungseffectes und Verschwinden nach 20' Erwärmen auf 100° zu verzeichnen. Analoge Versuche verliefen in der Regel auf die gleiche Weise.

Hemmung durch Organextracte.

Für die Herstellung der Organextracte wurden die Tiere durch Halsschnitt weitgehendst entblutet. Von den Organen wurden im Mörser 20%ige Suspensionen in physiologischer Kochsalzlösung hergestellt, die 24 Stunden im Eisschrank bewahrt wurden. Hierauf wurden die Zellen abcentrifugiert, und die überstehende Flüssigkeit wurde im Hemmungsversuch untersucht. In der Tabelle 3 sind die Ergebnisse von 2 Versuchen mit Meerschweinchen- und 4 Versuche mit Mäuse-Organextracten zusammengestellt, von letzteren in 2 Fällen auch mit erwärmten Organextracten.

Von den beiden Meerschweinchen zeigten die Herzextracte keine hemmende Wirkung in den untersuchten Concentrationen, auch die Lungenextracte waren nur schwach wirksam. Gut wirksam erwiesen sich die Gehirnextracte.

Ebenso wie bei den beiden Meerschweinchen zeigten bei den Mäusen 2 Herzextracte in den untersuchten Concentrationen keinen hemmenden Einfluss. Alle 4 Milzextracte wirkten gut, von den 4 Gehirnextracten 3.

Bei der Erwärmung der Organextracte von Maus 3 und 4 zeigte es sich, dass hierdurch die Hemmungswirkung zwar abgeschwächt, in keinem Fall aber aufgehoben wurde. In keinem Fall wurde die Hemmung verstärkt.

Weiterhin wurden von Mäusegehirn und Mäuseleber 20%ige Extracte mit Aethylalcohol hergestellt und soweit eingedampft, dass 1 ml Extract 1 g Organgewicht entsprach. Diese Extracte hemmten niemals.

Hemmung durch Hühner-Eiklaar.

Hühnereiweiss wies eine sehr starke Hemmung auf die Haemagglutination auf. Es zeigten sich zwar deutlich Unterschiede zwischen dem Material verschiedener Eier, aber in der Regel riefen Endverdünnungen von 1/1600—1/6400 noch complette Hemmungen hervor. Die Hemmung war auch bei Verwendung von mehr als der 4-fach haemagglutinierenden Dosis sehr kräftig.

Durch Erwärmen auf 56°, 63°, 70°, 80° wurde weder eine nennenswerte Wirkungssteigerung noch Wirkungsabschwächung erzielt,

TABELLE 3.

Hemmung der Haemagglutination von Hühnerblut durch *E. coli*, Stamm 65, durch Organextracte von Meerschweinchen und Maus.

Hemmung durch Organ-extracte	Meer-schwein-chen 1	Meer-schwein-chen 2	Maus 1	Maus 2	Maus 3			Maus 4				Maus 5 alcohol. Extract
					nativ	56°30′	70°15′	nativ	70°30′	80°15′	100°45′	
Gehirn	1/320	1/160	1/320	1/40	1/320	1/320	>1/40	1/320	1/160	1/320	1/80	> 1/20
Lunge	1/40	1/40	1/160	1/40								
Milz	1/80	1/40	1/320	1/320	1/640	1/320	1/80	1/640	1/160	1/160	1/160	
Leber	1/160	1/80	1/40	1/40	1/160	1/160	1/40	1/320	1/160	1/80	1/160	> 1/20
Niere	1/80	1/80	1/80	1/80								
Herz	>1/40	>1/40	>1/40	>1/40								

sogar 60 Minuten langes Kochen war ziemlich ohne Einfluss auf den Hemmungstiter. In einem Versuch hemmte beispielsweise natives Hühner-Eiklaar in der Verdünnung von 1/6400, nach 30′ 100° in einer solchen von 1/6400 und nach 60′ 100° in einer Verdünnung von 1/3200.

Verschiedene Versuche, den hemmenden Factor aus dem Eiklaar zu entfernen, erwiesen sich als vergeblich. In der Tabelle 4 ist ein Teil dieser Versuche wiedergegeben: Eine Lösung von 4% Hühner-Eiklaar in physiologischer Kochsalzlösung wurde mit 20% n HCl, 10% Acidum aceticum glaciale, 10% Esbach-Reagens, 10%, 30% und 50% Aethylalcohol, 10%, 30% und 50% Methylalcohol, 1% Formalin und 20% Kochsalz (cryst.) versetzt und 24 Stunden im Eisschrank bewahrt. Hierauf wurden die sauren Lösungen neutralisiert, und aufgetretene Niederschläge wurden abcentrifugiert. Die klaren Flüssigkeiten wurden im Hemmungsversuch geprüft. Es zeigte sich, dass nur durch 50% Aethylalcohol und 50% Methylalcohol eine merkliche Abschwächung eingetreten war, verschwunden war die Hemmungswirkung aber keineswegs. Die Alcoholniederschläge waren wirkungslos.

TABELLE 4.

Hemmung der 4-fach agglutinierenden Dosis von *E. coli*, Stamm 65, gegenüber Hühnerblut durch chemisch vorbehandeltes Hühner-Eiweiss.

Vorbehandlung von Hühnereiweiss durch:	Haemagglutinationstiter:
nativ	1/3200
10% Methylalcohol	1/1600
30% ,,	1/1600
50% ,,	1/800
hiervon Niederschlag	1/100 negativ!
10% Aethylalcohol	1/3200
30% ,,	1/3200
50% ,,	1/400
hiervon Niederschlag	1/100 negativ!
20% normal HCl	1/3200 ¹)
10% Acid. aceticum	1/1600 ¹)
10% Esbach-Reagens	1/1600 ¹)
20% NaCl	1/1600
1% Formalin	1/1600

¹) nach Neutralisierung.

Besprechung der Ergebnisse.

Besonders interessant ist der hemmende Factor im normalen Hühner-bzw. Menschenserum. Dieser erwies sich nicht nur als ziemlich weitgehend thermostabil, sondern es zeigte sich auch im Anschluss an das Erwärmen fast stets eine deutliche Zunahme des Hemmungstiters. Die Regelmässigkeit dieser Erscheinung, zusammen mit dem völligen Verschwinden der Hemmung nach 15—20′ Kochen, erweckt den Eindruck, dass es sich nicht um einen einfachen, sondern um einen complexen Factor handeln dürfte. Die eigentlich hemmende Componente ist relativ thermostabil, und wird normalerweise durch eine thermolabilere, antagonistisch wirkende zweite Componente in ihrer Wirkungskraft beschränkt. Durch kurzdauerndes Erwärmen wird dieser Antagonist zerstört, und somit steigt die hemmende Wirkung der thermostabilen Componente. Erst durch längeres Erwärmen wird auch diese schliesslich vernichtet.

Der Hemmungsfactor in den Mäuse- und Meerschweinchenorganextracten ist sicher mit dem im Normalserum von Mensch und Huhn vorhandenen nicht identisch, da er sich dem Erwärmen gegenüber anders verhält. Er gleicht viel eher dem wirksamen Hemmungsfactor im Hühner-Eiklaar.

Zusammenfassung.

Die Haemagglutination von Hühner-Erythrocyten durch *E. coli* (Stamm 65) wird durch Urin, Normalsera von Huhn und Mensch, durch Organextracte von Maus und Meerschweinchen und durch Hühner-Eiklaar gehemmt. Bei der Serum-Hemmung scheint der wirksame Factor seinerseits durch eine thermolabile antagonistische Componente beeinflusst zu werden. Aus Hühner-Eiklaar liess sich der hemmende Factor, der weitgehend thermostabil ist, durch chemische Einwirkung in keinem Fall völlig entfernen.

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BACTERIEN-HAEMAGGLUTINATION

III. DIE HEMMUNG DER COLI-HAEMAGGLUTINATION DURCH MANNOSE [1]

von

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(Eingegangen am 15. Juli 1954).

Einleitung.

Zum Verständnis der Zellreceptoren, die mit Microorganismen Verbindungen einzugehen vermögen, ist die Kenntnis der sogenannten unspecifischen Hemmungsstoffe von Bedeutung. Vor allem sind es bestimmte Polysaccharide, die bei der Virus-Haemagglutination genauer untersucht sind. Ueber die Wirkung der einfachen Kohlehydrate liegen anscheinend noch keine Untersuchungen vor.

Da die Haemagglutination durch den Stamm ,,65'' von *E. coli* nach Collier und Mitarbeiter (1955) ein ausgezeichnetes Modell für Hemmungsversuche darstellt, wurde für die vorliegenden Versuche dieser Coli-Stamm benutzt. Die angewandte Technik ist die gleiche, die in den erwähnten Arbeiten beschrieben wurde.

Die benutzten Kohlehydrate.

Bei Glucose, Lactose und Saccharose handelt es sich um Praeparate der Pharmacopee. Die Maltose stammte von Brocades Stheeman, Mannit und Mannose von der Pfanstiehl Chemical Co., Arabinose, Dulcit, Galactose und ,,d-Sorbit'' von der Fisher Scientific Co., und Adonit, Erythrit, Inosit, Raffinose, Rhamnose, Sorbit, Trehalose und Xylose von der Schering-Kahlbaum A.G. aus der Zeit vor dem Kriege.

Hühnererythrocyten-Agglutination durch Kohlehydrate.

Zunächst musste festgestellt werden, ob die uns zur Verfügung

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1) Teil I und II: Antonie van Leeuwenhoek **21**, 113, 124, 1955.

stehenden Kohlehydrate selber die Eigenschaft besässen, Hühnererythrocyten zu agglutinieren. Zu diesem Zweck wurde eine 20%ige Lösung von ihnen in Aq. dest. hergestellt und hiervon ausgehend weitere Verdünnungen mit phys. Kochsalzlösung. Zu je 0,5 ml dieser Verdünnungen kam 0,5 ml Hühnererythrocyten-Suspension. Nach 1½—2 Stunden bei Zimmertemperatur wurde abgelesen. Die Ergebnisse sind in der Tabelle 1 zusammengestellt.

TABELLE 1.

Haemagglutination von Hühnerblut und Hemmung der Haemagglutination von Hühnerblut mittels *E. coli*, Stamm 65, durch Kohlehydrate.

Kohlehydrat:	Haemagglutination bei Endverdünnung:				Hemmung der Haemagglutination:
	1/10	1/20	1/40	1/80	
Adonit	0	0	0	0	0
Arabinose	0	0	0	0	0
Dulcit	+++	+	0	0	0
Erythrit	(+)	0	0	0	0
Galactose	0	0	0	0	0
Glucose	0	0	0	0	+
Inosit	0	0	0	0	0
Lactose	0	0	0	0	0
Mannit	+++	+++	0	0	0
Mannose	Lyse	Lyse	(+)	0	+++!
Maltose	+++	+	0	0	0
Raffinose	+++	+	(+)	0	0
Rhamnose	0	0	0	0	0
Saccharose	0	0	0	0	(+)
Sorbit	+	0	0	0	0
d-Sorbit	+++	+++	+++	+++1)	0
Trehalose	(+)	0	0	0	+
Xylose	+	0	0	0	0

1) bis 1/320 = +++ ; 1/640 = 0.

Es zeigte sich, dass Mannose in den Endconcentrationen von 1/10 und 1/20 haemolysierte und in der Endconcentration von 1/40 schwach agglutinierte. In anderen Versuchen war die Haemolyse nur gering. Dulcit, Mannit, Maltose und Raffinose agglutinierten kräftig, Erythrit, Sorbit, Trehalose und Xylose nur schwach, Galactose nur andeutungsweise. Eigenartig war das Verhalten des „d-Sorbits", das bis zur Verdünnung von 1/320 stark agglutinierte.

Hemmung durch Kohlehydrate.

Weiterhin wurde mittels des Hemmungsversuches der Einfluss der verschiedenen Kohlehydrate (in selber nicht agglutinierender Dosierung) auf die Haemagglutination von Hühnererythrocyten durch Stamm 65 in vierfach wirkender Dosis untersucht. Auch diese Resultate finden sich in Tabelle 1 zusammengestellt. Durch Trehalose wurde eine mässige Hemmung erzielt, durch Saccharose eine sehr schwache. Dagegen war Mannose imstande, die Haemagglutination sehr stark zu hemmen. Alle anderen Kohlehydrate erwiesen sich als wirkungslos.

Hemmung durch Mannose.

Die Hemmung durch Mannose erwies sich als ausserordentlich stark. Nicht allein die 4-fach agglutinierende Dosis der Bacteriencultur gegenüber Hühnerblut wurde gehemmt, sondern auch erheblich höhere Multipla.

TABELLE 2.

Hemmung der Haemagglutination von Hühnerblut durch *E. coli*, Stamm 65, durch Mannose.

Multiplum der einfach haemagglutinierenden Dosis:	Versuch 1: Mannose nativ	Versuch 2:	
		Mannose nativ	Mannose 100° 60′
1 ×	1/2048000	1/256000	1/512000
2 ×	1/256000	1/256000	1/512000
4 ×	1/128000	1/256000	1/256000
8 ×	1/64000	1/128000	1/256000
16 ×	1/64000	1/128000	1/64000
32 ×	1/32000	1/64000	1/32000
64 ×	1/32000	1/64000	1/32000

In der Tabelle 2 sind zwei Versuche wiedergegeben, aber zahlreiche weitere Versuche mit jungen und alten Culturen verliefen völlig analog. Im 1. Versuch wurde die einfach haemagglutinierende Dosis noch durch eine Mannoseverdünnung von 1/2.048.000 gehemmt, und die 64-fache Dosis durch eine Mannoseverdünnung von 1/32.000. Im 2. Versuch wurde nebeneinander eine frisch bereitete und eine 1 Stunde lang gekochte Mannoselösung untersucht. In beiden Fällen wurde die 4-fach agglutinierende Bacteriendosis durch eine Mannose-Endconcentration von 1/256.000 (= 4 Gamma) gehemmt.

Diese Hemmung der Haemagglutination durch Mannose wurde nicht nur gegenüber nativen Bacterienculturen festgestellt, sondern auch gegenüber 30 Minuten auf 56° erwärmte Bacterien und auch gegenüber durch Centrifugieren bacterienfrei gemachte Media. Hier wurden stets analoge Werte gefunden.

Weiterhin war die gleiche Hemmung zu beobachten, wenn statt Hühnerblut Erythrocyten von anderen Tierspecies benutzt wurden. So hemmt beispielsweise Mannose die Haemagglutination von Stachelschweinerythrocyten durch die 4-fach agglutinierende Bacteriendosis in einer Endconcentration von 1/51.200. Versuche mit Meerschweinchenerythrocyten ergaben analoge Werte.

Bindung von Mannose an die Erythrocyten.

Zur Feststellung, ob die Mannose an die Erythrocyten adsorbiert wird, wurde eine 20%ige Suspension gewaschener Hühnererythrocyten in einer 1%igen Mannoselösung je 1 Stunde lang bei 4°, bei Zimmertemperatur und bei 37° bewahrt. Hierauf wurden die Erythrocyten 5-mal mit Kochsalzlösung ausgewaschen. Diese vorbehandelten Erythrocyten nebst unvorbehandelten Controllerythrocyten wurden nunmehr zur Bestimmung des Haemagglutinationstiters einer Bacteriencultur benutzt. In allen 4 Versuchen wurde der gleiche Endtiter gefunden, sodass man annehmen darf, dass nach dem Auswaschen keine Mannose mehr an den Erythrocyten adsorbiert sein konnte.

Weiterhin wurden die drei überstehenden Flüssigkeiten der drei Blut-Mannose-Suspensionen, ferner die überstehende Flüssigkeit einer analog hergestellten und 24 Stunden bei 4° aufbewahrten Blut-Mannose-Suspension und schliesslich die 1%ige Mannoselösung allein auf Hemmungswirkung untersucht. Die 3 eine Stunde lang gebundenen Flüssigkeiten und die Mannoselösung hemmten die Coli-Haemagglutination bei einer Mannose-Endverdünnung von 1/102.400, die 24 Stunden bei 4° gebundene Flüssigkeit bei einer solchen von 1/51.200. Von der zu den Erythrocyten gefügten Mannoselösung war also practisch nichts an die Erythrocyten gebunden worden.

Bindung von Mannose an die Bacterien.

Um festzustellen, ob Mannose mit den Bacterien eine feste Bindung eingeht, wurde folgender Versuch angestellt:

Die von 5 ml Bouilloncultur abcentrifugierten Bacterien wurden in 5 ml Mannoselösung 1/1000 in Kochsalzlösung resuspendiert und 1 Stunde bei 37° und in einem weiteren Versuch bei 4° bewahrt. Hierauf wurden die Bacterien wieder abcentrifugiert. Die überstehende Flüssigkeit ergab in der Endverdünnung von 1/256 völlige Hemmung der Haemagglutination. Die Bacterien wurden noch zweimal mit je 5 ml Kochsalzlösung ausgewaschen, und beide Waschwässer ergaben in der Endverdünnung von 1/8 keine Hemmung mehr.

Ein Controllversuch, bei dem abcentrifugierte Bacterien in Kochsalzlösung suspendiert und 1 Stunde bei 37° bzw. 24 Stunden bei 4° belassen waren, ergab bei Untersuchung der überstehenden Flüssigkeit und der beiden Waschwässer in keinem Fall Hemmung. Die mit Mannose vorbehandelten und zweimal ausgewaschenen Bacterien wurden ebenso wie analog mit Kochsalzlösung vorbehandelte Controll-Bacterien in 5 ml Kochsalzlösung suspendiert und in fallenden Verdünnungen auf ihre haemagglutinierende Fähigkeit geprüft. Die mit Mannose vorbehandelten Bacterien ergaben einen Endtiter von 1/256, die mit Kochsalzlösung vorbehandelten einen Titer von 1/128, und eine frische Controllcultur einen solchen von 1/512.

Man darf hieraus den Schluss ziehen, dass die Mannose durch die Bacterien nicht fest gebunden wird, sondern leicht mit physiologischer Kochsalzlösung auszuwachsen ist.

Bindung von Mannose an Bacterien + Blut.

Es blieb noch zu untersuchen, ob nicht bei dem Vorhandensein von Bacterien und Blut gemeinschaftlich doch eine feste Bindung der Mannose zustande käme. Zu diesem Zweck wurden je 2 ml einer 1%igen Mannoselösung mit je 1 ml unverdünnter Cultur + 1 ml 10%iger Erythrocytensuspension, im andern Fall mit 1 ml Cultur + 1 ml Kochsalzlösung, im weiteren Fall mit 1 ml Blutsuspension + 1 ml Kochsalzlösung und im letzten Fall mit 2 ml Kochsalzlösung gemengt und 2 Stunden lang bei 37° gebunden. Hierauf wurde scharf abcentrifugiert und die überstehenden Flüssigkeiten nach 24 und 48 Stunden Aufenthalt im Eisschrank im Hemmungsversuch austitriert. Im ersten und zweiten Versuch wurden in alle 4 Fällen die gleichen Titer gefunden, wie aus Tabelle 3 hervorgeht.

TABELLE 3.

Bindung von Mannose an *E. coli*, Stamm 65 + Hühnererythrocyten.

Gemisch von:				Ueberstehende Flüssigkeit:	
Mannose 1/100	Voll-Cultur	Hühner-blut 10%	NaCl-Lösung	Titer nach 24 Std.	Titer nach 48 Std.
2,0	1,0	1,0	—	1/51200	1/51200
2,0	1,0	—	1,0	1/51200	1/102400
2,0	—	1,0	1,0	1/51200	1/51200
2,0	—	—	2,0	1/51200	1/51200

Analoge Versuche mit 0,1%iger Mannoselösung ergaben die gleichen Resultate. In keinem Fall war die Mannoseconcentration durch die Erythrocyten, durch die Bacterien oder durch die Combination Bacterien + Blut nachweisbar vermindert.

Wurden Haemagglutinationsreihen angesetzt und nach Beendigung der Reaction wieder aufgeschüttelt, so konnte eine nunmehrige Beigabe von Mannose eine erneute Agglutination entweder völlig oder doch wenigstens teilweise hemmen. Durch das Aufschütteln wurde also mindestens teilweise die Bindung zwischen Agglutinin und Erythrocyten gelöst und das Eingehen einer erneuten Bindung durch die Mannose verhindert.

Fehlender Einfluss von Mannose auf die Vaccine-Haemagglutination.

Von einem Schaf[1]) dem mit Vaccinevirus inficiertes Schaf-Embryonalmaterial subcutan implantiert worden war, wurde nach 72 Stunden etwas Exsudat gewonnen, das in der Verdünnung von 1/64 Hühnererythrocyten agglutinierte. Bei keiner Virusconcentration übte Mannose in der Endconcentration von 1/400 den geringsten Einflus aus.

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[1]) Hier sei kurz erwähnt, dass die Schafrasse von Suriname haemagglutinierende Ovine lieferte, während dies bei den indonesischen Schafen nicht der Fall war. Auch die Pulpaausbeute war in Suriname drei- bis viermal so gross wie in Indonesien.

Fehlender Einfluss von Mannose auf die Col. S.K. Virus-Haemagglutination.

Die Haemagglutination von Hammelblut durch das Col. S. K.-Virus wurde bei keiner Virusconcentration durch Mannose in einer Endconcentration von 1/400 gehemmt.

Fehlender Einfluss von Mannose auf die Immun-Haemagglutination und Immun-Haemolyse.

Für die Versuche wurde ein Anti-Hammelblut-Kaninchenserum von geringem Titer (1/1000) als Amboceptor und 24 Stunden altes Meerschweinchen-Complement benutzt.

Fallende Verdünnungen von Mannose (1/100, 1/200 etc.) wurden mit 1/200 Amboceptorverdünnung 1 Stunde im Wasserbade bei 37° gebunden. Hierauf wurde 2%ige Hammelblutsuspension zugefügt und wieder ins Wasserbad gebracht. In jedem Fall kam es zu starker Haemagglutination.

Fallende Verdünnungen von Mannose (1/100, 1/200 etc.) wurden mit 1/200 Amboceptorverdünnung gemengt und 1 Stunde bei 37° im Wasserbad gebunden. Hierauf wurde Complement 1/30 und 2%ige Hammelblutsuspension zugefügt und wieder ins Wasserbad gebracht. In jedem Fall kam es zu completter Haemolyse.

Fallende Amboceptordosen (1/20, 1/40 etc.) wurden mit einer constanten Verdünnung von Mannose (1/200) gemengt und eine Stunde im Wasserbad gebunden. Ebenso wurde eine Amboceptor-Verdünnungsreihe ohne Mannose vorbehandelt. Hierauf wurde 2%ige Hammelblut-Suspension zugefügt. In beiden Versuchsreihen zeigte sich complette Haemagglutination bis zur Amboceptorverdünnung von 1/640.

Fallende Amboceptordosen (1/20, 1/40 etc.) wurden mit einer constanten Verdünnung von Mannose (1/200) gemischt und eine Stunde lang im Wasserbade gebunden. Eine weitere Reihe ohne Mannose wurde analog behandelt. Hierauf wurde Complement 1/30 und 2%ige Hammelblut-Suspension zugefügt. Beide Reihen ergaben bei der Amboceptorverdünnung von 1/640 complette und in der Verdünnung von 1/1280 deutliche Haemolyse.

Fallende Verdünnungen von Mannose (1/100, 1/200 etc.) wurden mit constanter Menge Complement (1/30) gemengt und 1 Stunde im Wasserbade gebunden. Hierauf wurde eine constante Menge

Amboceptor (1/200) und 2%ige Hammelblut-Suspension zugefügt. In jedem Fall trat complette Haemolyse auf.

Fallende Verdünnungen von Complement (1/10, 1/20 etc.) wurden mit einer constanten Menge von Mannose (1/200) gemengt und 1 Stunde im Wasserbad gebunden. Hierauf wurde eine constante Menge von Amboceptor (1/200) und 2%ige Hammelblut-Suspension zugesetzt. Das gleiche war der Fall bei einer Controllreihe von Complement-Verdünnungen ohne Mannose. In beiden Reihen kam es bis zur Complementverdünnung von 1/80 zu kompletter Haemolyse, bei höheren Complementverdünnungen zu Haemagglutination.

Fasst man alle diese Beobachtungen zusammen, so zeigt sich, dass Mannose auf die Immun-Haemagglutination und die Immun-Haemolyse keinerlei Einfluss auszuüben vermag, ganz gleich, welche Componente des Systems vorher gebunden wurde.

Zusammenfassung.

Mannose wirkt in einer Endconcentration von 1/20 auf Hühnerblut mehr oder weniger haemolytisch, in einer solchen von 1/40 leicht agglutinierend.

Während durch Trehalose und Saccharose eine leichte Hemmung der Agglutination von Hühnerblut durch den Stamm 65 von *E. coli* verursacht wird, ist die Haemagglutinations-Hemmung durch Mannose ausserordentlich stark. Die 4-fach haemagglutinierende Bacteriendosis kann durch eine Endconcentration von 1/256.000 Mannoselösung gehemmt werden.

Die Bindung der Mannose an das System Blut-Bacterien scheint sehr locker zu sein.

Die Vaccine- und Parapoliomyelitis-Haemagglutination wird durch Mannose nicht gehemmt, ebensowenig die Immun-Haemagglutination und Immun-Haemolyse.

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(From the National Institute of Public Health, Utrecht).

SOME REMARKS ON *SALMONELLA PARATYPHI* TYPE HEIDELBERG

by

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CLINICAL OBSERVATION.

C.L. a female baby aged 3 months entered into hospital while suffering from a still growing angioma cavernosa occupying the left labium majus. The surgeon asked pediatrical advice before operation, because the baby suffered from slight fever, serious diarrhea with mucosanguineous motions.

C.L. is the third child of a healthy but nervous mother and a father suffering from stomach ulcer treated by medicine and diet. He kept his diet very strictly helped by his wife, who from sheer fear was very careful in preparing food for the whole family. They never used canned meat, sausages, egg powder, duck eggs, never kept house pets or fowls and stayed always at home without guests to avoid temptation to change food and drinks.

R.I.V. cultures revealed the presence of *Salmonella paratyphi* type Heidelberg in the feces. After a week of dietical treatment and 500 mg sulfaguadinin every 24 hours the diarrhea diminished a little, but growth was very insufficient and culture of the stools still contained *S. paratyphi* type Heidelberg. In stead of sulfoguadinin chloromycetin now was administered. A week later the whole condition was unchanged, the feces still positiv for *S. paratyphi* type Heidelberg. As the angioma was very rapidly growing, the operation was carried out and was very successful. The wound was not contaminated, but the stools still contained *S. paratyphi* type Heidelberg. This strain has been tested on resistence. Chloromycetin was active in a concentration of 5—10 γ, tetracyclin in one of 2—5 γ.

Treatment by chloromycetin was stopped now and neomycin

(Lundberg) was supplied for 7 days in a dose of 75 mg every 24 hours. The general condition was improving, but diarrhea increased after a few days. After this week stools were free from *Salmonella* and baby went home in a fairly good condition.

In order to trace the way of infection the whole family has been examined. We found out that an elder brother had suffered from diarrhea a fortnight earlier than our baby patient. His stools were free from *Salmonella* like those of the whole family, but his agglutination titer was 1/50 against *S. paratyphi* type Heidelberg just as it was in our little patient. The only hygienic mistake we could spot with the mother's help was that she had once used rain-water collected from eaves gutters soiled by birds droppings to wash baby's clothes and napkins. In bacteriological control the rain-water proved sterile for *Salmonella*.

S. paratyphi type Heidelberg belongs to the group of paratyphus B with the following antigen formula: IV, V, XII, I, 2, (3) and was found for the first time in Heidelberg (1933) by HABS (1) during an epidemic of gastroenteritis after the slaughtering of pigs. The patients — 19 in all — had consumed liver sausage and "Schwartenmagen". One female died, one child was seriously ill.

BOECKER (1935) found *S. paratyphi* type Heidelberg in Kassel's public slaughterhouse after food poisoning by saveloy (1).

HOHN and HERRMANN (1933—1939) examined 386 *Salmonella* strains and found one case of *S. paratyphi* type Heidelberg and 3 of *S. bovis morbificans*.

In the reports of the National Health Service of the Netherlands East Indies (2) from 1930 till 1938 *S. paratyphi* type Heidelberg is twice mentioned in human patients, but ERBER (3) isolated already 20 strains from humans. Cultures from 150 eggs brought for examination revealed the presence of *Salmonella* in 25, *S. paratyphi* type Heidelberg was present in 2.

KAUFFMANN (1952) found 4 times *S. paratyphi* type Heidelberg in Belgian Congo, twice in children with gastroenteritis, once in an obviously healthy adolescent and once in a guinea pig.

From 1945 till 1953 only two human cases of *S. paratyphi* type Heidelberg were found in the National Health Institute, Utrecht, but 1954 was characterized by an explosion of 24 cases between April and July, spread over the country with only a few places with more than one case. In none of these cases any relation between kind of food or drink could be traced and as suddenly as it had appeared this *Salmonella* wave had disappeared.

Dr Bijlmer (Deventer) furnished information about his cases. The first one appeared in a hospital in which some patients with *S. Schottmülleri* had been nursed. One of the nurses taken up in the surgical ward for treatment of piles suffered from fever and diarrhea due to *S. paratyphi* type Heidelberg. A month later some persons in Zwolle fell ill with high fever, headache, diarrhea and lumbar ache in who a treatment with sulfonilamides failed. The first one suffered from *S. typhi murium*, the second from *S. paratyphi* type Heidelberg like his son (3rd case). The 4th and 5th cases were brothers. There had not been any contact between these families. A butcher's family might have been the "missing link", but neither bacteriological, nor serological proof could be obtained.

In another family the mother was a carrier of *S. bareilly*, two children suffered from *S. paratyphi* type Heidelberg, whilst a third one harbored both *S. paratyphi* type Heidelberg and *Shigella flexneri*.

Another curious case was a female patient nursed for some weeks in a hospital for neurosis, eating and drinking the same as the other patients, who complained of fever and diarrhea during two days caused by *S. paratyphi* type Heidelberg.

In any case of explosive food poisoning it is not only needed to find out the cause, mostly *Salmonella*, but to classify the strain as completely as possible with the assistance of the modern *Salmonella* Centers (Utrecht, New York, Berlin) in order to spot as soon as possible the source of contamination. Nowadays every student of salmonellosis is convinced that apparently healthy animals, pigs, turkeys, fowls, ducks, cows may be infected by *Salmonella*. Even in situations far away from such *Salmonella* Centers a more correct diagnosis will be possible thanks to the "drying method" (4) by which stool samples can be sent over.

For treatment of salmonellosis type Heidelberg the sulfonamides seem without any influence, streptomycin is acting in vitro, auromycin is less successful than chloromycetin whilst neomycin and tetracyclin stand at the top.

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YEASTS IN SOME NETHERLANDS SOILS

by

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INTRODUCTION [1]).

In the circulation of yeasts in nature the soil plays a very important part. The researches of PASTEUR (30), HANSEN (13, 14, 15, 16), MÜLLER-THURGAU (28), BERLESE (2), WORTMANN (33), BOUTROUX (5), CORDIER (6) and HEINZE (21) have shown indeed that yeasts from the surface of mature fruits on which they multiply in large numbers, reach the soil by means of rain and also by the dropping of the fruits.

Yeasts, therefore, are most frequently met with in samples of soils from vine-yards and orchards or from neighbouring lands. In 67% of these HANSEN (17, 18) detected yeasts in contrast to samples from other soils, such as woodlands, in which he ascertained their presence to the extent of 30%. The distribution of yeasts, however, is brought about by other agents, such as: wind, insects visiting flowers and fruits, manuring with wine dregs and other residues of alcohol fermentation, which favour strongly the multiplication of yeasts and manuring with dung and other faecal material, as these micro-organisms may pass through the digestive tract of animals uninjured. Hence the possibility of meeting yeasts also in areas distant from fruit-producing plants, where HANSEN found them to an extent of 19% of the samples. Their number is maintained at a relatively high level during the period of fruit ripening, i.e., in summer and in autumn. It tends to be reduced during winter and they are present in still smaller numbers in spring. On studying their survival in soils, Hansen (19, 20) noted that in soils inoculated with *Kloeckera apiculata* and kept under natural conditions, this species could be recovered three years after inoculation, while spore-forming yeasts, such as *Saccharomyces ellipsoideus*, *S. pastorianus* and other beer yeasts maintained their number unaltered for at least a year, after which, although decreased in number, they could survive for a much longer period than non-sporeforming yeasts.

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[1]) Since in this Review it is impossible to quote references completely owing to lack of space, all references are inserted in another work, entitled "Yeasts in some Italian soils" which appeared in "Rivista di Biologia, Perugia (Italia)" **47, 2, 1955.**

The survival of yeasts, however, is not to be considered in relation to the capacity of maintaining a latent state of life, only, but also to suitable conditions of food, humidity and temperature permitting their development.

Müller-Thurgau (28) was able to show that in vine-yards and orchards, yeasts could be recovered at depths of 30 up to 40 cm. On the surface of unripe fruits, not finding suitable nourishment, the yeasts remain in a latent state of life or die suddenly from adverse conditions such as sunlight, desiccation, etc. But when they happen to be present upon ripe fruit they undergo an active process of multiplication, particularly in the lesions of the skin, effected continually by birds, insects, weather, etc. These lesions inevitably produce, even though in small quantities, a sugary juice, which runs out and encourages the rapid and abundant prolification of micro-organisms which after the fruit season is over, are destined to inhabit the soil once again.

Berlese (3) stressed the action of insects which would not only act as a vehicle of transport, but also promote conservation: in fact yeasts can be found in the intestines of some *Dipterae* together with sugary material suitable for optimum proliferation, thus offering favourable conditions for passing the winter.

Fischer (11) found yeasts to be particularly abundant in heather-bearing soil and their number to increase in soils rich in humus.

Ciferri (7) noticed that in lands given over to vine-yards yeasts are represented by double the number of those found unsuitable for planting vines. In land cultivated for many years with forage grasses, only 5 to 6 cells of yeasts per g of soil could be found. Two species of *Saccharomyces* and only one of *Torulopsis* have been studied, Ciferri carrying out inoculation tests into the soil they were derived from. Initially he obtained a slight increase in the number of cells, but after 10 days, a rapid decrease in number occurred. After two months only 29%—10% of the number of cells originally introduced into the soil could be recovered. He ascertained that by the action of rainwater only a minute quantity of yeasts is capable of percolating through 6 cm of humus soil; such a quantity, however, does not influence the decrease in yeasts content of the soil. Besides CIFERRI noted that in the soil the actively sporing yeasts have a greater vitality when compared with the non-sporing types, confirming thereby the observation of DE KRUIFF (22), who had found that in soil the latter constitute a small percentage of the number of spore-forming yeasts.

In 1928 CIFERRI studied soils from the Dominican Republic and noted that the quantity of yeasts was very small when compared with *Eumycetes* and *Schizomycetes* (8). Vegetative cells of yeasts appeared to have died off within a week after having been introduced into the soil.

LOCKHEAD and FARREL (23) in the scope of clarifying the relationship between soil and the yeasts occurring in honey, arrived at the conclusion that the osmophilic yeasts occur exclusively in soils near beehives. They isolated 175 strains belonging to 7 different species.

In Germany NISSEN (29) during 4 seasons of the year took 180 samples belonging to 10 chemically and physically different soil types in 50% of which he detected yeasts. The largest number of yeasts were found in fruit-bearing lands, whilst the lowest content was found in wooded land. The yeasts belonged to the genera *Saccharomyces, Hansenula, Candida, Torulopsis* and *Rhodotorula.*

BOUTHILET (4) detected yeasts in 48 soils of Minnesota sampled in spring, over 80% of the 117 cultures isolated belonging to the genera: *Debariomyces, Torulaspora, Mycoderma, Pichia, Torulopsis, Trichosporon* and *Schizoblastosporon.*

According to LUND (26) the number of yeasts in Danish soils varies from only a few to some thousands per g. They were more numerous on the surface of the soil, but were to be found in smaller numbers at greater depths up to 30 cm. There was no obvious numerical difference between the yeasts present in cultivated and uncultivated soils. In the latter the number of yeasts decreases more rapidly than in the former with increasing depth. The species isolated belong to the genera *Hansenula, Candida* and *Torulopsis.* No substantial differences were noticed in the various strata of the soil.

THE SCOPE OF THE PRESENT INVESTIGATION.

The foregoing information, while demonstrating the notable influence of the soil on the natural distribution of yeasts, is far from being sufficient in elucidating the real function of the yeasts. On the other hand, although they are very scarce in number when compared with other microbes of agronomic interest, it is not possible to regard the yeasts simply and solely as occasional inhabitants of the soil. In particular there still is much confusion about the variation of the species of yeasts found in the soil and those that develop on grapes and afterwards in the must on conservation. For our present knowledge of the influence exercised by the climate on the quantitative composition of the flora of *Saccharomycetes* in soils of various nature and in the presence or absence of vines needs still elucidation.

In the scope of clarifying some of these factors in 1952 we started a series of experiments on soils under completely different climatic and agronomic conditions. In fact we have taken into consideration soils of central Italy under cultivation with vines or about to be cultivated with them and Netherlands soils in which the vine has never been cultivated. In the present paper we report the results obtained on the flora of *Saccharomycetes* of some Netherlands soils.

Soils studied.

12 soils, all of them taken from ground of the Laboratory of Microbiology of the Agricultural University of Wageningen, were considered. 10 samples were derived from 10 plots in the experimental field, while 2 were taken from the neighbouring orchard.

The experimental field is composed of 40 plots, layed out in 5 rows which are separated by paths 1 m wide. Each plot of 42 m^2 is enclosed by low walls which rise 20 cm above the surface of the soil and descend into the soil for another 75 cm, so as to reach the level of the inert layer, which, however, in 1921 constituted the actual surface of the ground. In that year the surface of the ground was levelled, the walls were constructed and the plots were prepared, bringing onto each a 75 cm layer of black soil and 15 quintals of highmore peat, which were incorporated in the top 15 cm of black soil. An active soil was thus artificially formed, which was put under cultivation the same year. To all plots, up to the present day, every year the same manuring has been applied, being different from plot to plot, and all have been cropped with the same plants, these crops varying in successive years.

The scheme of manuring may be seen from the following list, in which the plots examined by me are arranged in progressive numbers, whilst the numbers in use at the Institute are added in brackets.

1 (40) Each year artificial manure without K.
2 (32) ,, ,, stable manure.
3 (8) ,, ,, artificial manure (K + N + P)
4 (39) ,, ,, as 3, but without N.
5 (31) ,, ,, as 3, but N in the form $(NH_4)_2SO_4$.
6 (38) No fertilization.
7 (30) Each year artificial manure as 3, but N in the form $NaNO_3$.
8 (3) In 1921, 60 kg of $CaCO_3$ and artificial manure each successive year.
9 (26) Artificial manure alternating with stable manure.
10 (1) Original sandy subsoil without black soil and without peat.
11 Soil taken in the proximity of an apple tree. Normal fertilization.
12 Soil taken in the proximity of a peach tree. Normal fertilization.

Experimental Methods.

The soils under consideration have been analysed twice. The first sampling of 1 g took place in the 3rd week of October. Since yeasts were found in only 50% of the samples it was thought opportune to repeat the analysis a few days later, using 4 g of soil. In both cases samples were taken at a depth of 5 cm, bringing about 50 g into sterile Petri dishes and carefully mixing them with a sterile rod. The samples of 1 g and 4 g were put into stoppered bottles of 50 ml, and these were immediately filled with a medium consisting of: water 100 ml, glucose 5 g, yeast extract 2 ml and citric acid to a pH of 3.5. The stoppered bottles were incubated at 30° C. After 24 hours microscopic examinations were started and repeated on successive days. Production of gas in the bottles was also recorded.

From positive cultures isolates were made on grape must gelatine, prepared immediately before use. The dishes were kept under observation for 15 days at 16—18° C. and from all types of colonies cultures were made.

From the two series 105 cultures of yeast have been isolated and their identification was performed at the Microbiology Institute of the University of Agriculture at Perugia.

Microscopic examination was carried out on young (2—3 days old) cultures in must and on malt agar, incubated at 28° C.

The cultural characteristics were determined on grape must and malt agar. The former were kept under observation for 30 days at 20° C., whereas the latter were observed for over 3 months at room temperature. Spores were looked for in old malt agar cultures, on Gorodkowa agar and on chalk blocks as described by HANSEN.

For the liquifaction of gelatine tubes with grape must gelatine were kept under observation for 75 days. The sugar fermentation was tried out in Durham test tubes, containing peptone broth with 2% of the sugar under consideration. Giant colonies were obtained by plating a small drop of a young culture in must onto the surface of grape must gelatine. The assimilation of nitrates and the development in the presence of ethyl alcohol as only source of carbon food were tried according to the method of STELLING-DEKKER (31). The assimilation of sugar was investigated according to the auxonographic method as advised by LODDER and KREGER-VAN RIJ (25). The fermentative power was determined on 50 ml of must, containing 29% of sugar introduced into a 100 ml Erlenmeyer flask. The precipitate formed during sterilization was never separated from the must and after inoculation the flasks were capped with a rubber stopper fitted with a Miller valve, then weighed and incubated at 20—25° C. The weighings were repeated at intervals of 5 days until after two successive weighings a constant weight was obtained. From the difference between the first and last weighings the amount of CO_2 given off was determined and the alcohol produced calculated from this figure.

For the identification of isolated species we were greatly helped by the works of LODDER (24), WICKERAM (32), CIFERRI and READELLI (9), LODDER and KREGER-VAN RIJ (25), GUILLIERMOND (12), STELLING-DEKKER (31), DIDDENS and LODDER (10), ARNAUD (1).

RESULTS.

In table 1 for the various soils tested the species isolated in the two analyses and the fermentative power of each culture are given.

From table 1 it can be inferred that very different results have been obtained by the two successive analyses. In fact the enrich-

TABLE 1.

Sample of soil	Analysis with one gram: 26.10.52. Species isolated:	Alcohol % vol.	Analysis with four gram: 14.11.52. Species isolated:	Alcohol % vol.
1	1 *Saccharomyces mangini*	10.3	39 *Kloeckera apiculata*	4.32
	2 ,, ,,	12.05	40 *Torulopsis inconspicua*	0.58
	3 ,, ,,	11.22	41 ,, ,,	0.50
	4 ,, ,,	10.50	42 *Kloeckera apiculata*	4.37
	5 ,, ,,	11.78	43 *Saccharomyces mangini*	10.65
	6 *Aureobasidium pullulans*	—	44 ,, ,,	11.02
	7 *Aureobasidium pullulans*	—	45 *Aureobasidium pullulans*	—
2			46 *Kloeckera apiculata*	5.00
			47 *Torulopsis inconspicua*	0.70
			48 ,, ,,	0.77
			49 ,, ,,	1.57
3	8 *Candida Guilliermondii*	5.35	50 *Kloeckera apiculata*	4.72
	9 ,, ,,	5.52	51 *Torulopsis inconspicua*	0.52
			52 ,, ,,	2.02
			53 *Kloeckera apiculata*	4.09
4			54 *Kloeckera apiculata*	5.77
			55 *Sacch. ellipsoideus*	12.95
			56 *Kloeckera apiculata*	4.42
			57 *Sacch. ellipsoideus*	11.99
			58 ,, ,,	11.77
			59 ,, ,,	12.00
5	10 *Aureobasidium pullulans*	—	60 *Kloeckera apiculata*	4.70
			61 *Hansenula saturnus*	1.07
	11 *Hansenula saturnus*	1.45	62 *Candida Guilliermondii*	4.15
	12 ,, ,,	1.45	63 ,, ,,	4.03
6			64 *Kloeckera apiculata*	5.25
			65 *Torulopsis inconspicua*	2.02
			66 ,, ,,	0.45
			67 ,, ,,	1.00
7			68 *Kloeckera apiculata*	4.50
			69 ,, ,,	4.60
			70 *Hansenula saturnus*	0.55
			71 ,, ,,	0.42
			72 ,, ,,	0.60
			73 ,, ,,	0.92
			74 ,, ,,	0.92

TABLE 1 (continued).

Sample of soil	Analysis with one gram: 26.10.52. Species isolated:	Alcohol % vol.	Analysis with four gram: 14.11.52. Species isolated:	Alcohol % vol.
8			75 *Kloeckera apiculata*	4.77
			76 ,, ,,	5.55
			77 *Torulopsis inconspicua*	0.17
			78 ,, ,,	0.28
9			79 *Hansenula saturnus*	0.85
			80 ,, ,,	0.78
			81 ,, ,,	1.68
10	13 *Hansenula saturnus*	0.85	82 *Kloeckera apiculata*	4.50
	14 ,, ,,	1.6	83 ,, ,,	5.40
			84 *Hansenula saturnus*	1.30
			85 ,, ,,	3.00
			86 ,, ,,	1.35
11	15 *Kloeckera apiculata*	4.90	87 *Kloeckera apiculata*	4.42
	16 ,, ,,	4.95	88 *Hansenula saturnus*	1.01
	17 *Hansenula saturnus*	1.18	89 ,, ,,	0.90
	18 ,, ,,	0.7	90 *Torulopsis pulcherrima*	1.20
	19 *Pichia membranefaciens*	0.47	91 ,, ,,	0.70
	20 *Kloeckera apiculata*	4.90	92 ,, ,,	1.00
	21 *Pichia membranefaciens*	0.7	93 *Pichia membranefaciens*	0.51
	22 ,, ,,	2.32	94 *Aureobasidium pullulans*	—
	23 ,, ,,	—	95 *Torulopsis pulcherrima*	0.93
	24 ,, ,,	—	96 *Pichia membranefaciens*	0.12
	25 *Kloeckera apiculata*	4.4	97 *Rhodotorula mucilaginosa*	—
	26 *Aureobasidium pullulans*	—		
	27 *Rhodotorula mucilaginosa*	—		
12	28 *Kloeckera apiculata*	4.38	98 *Kloeckera apiculata*	5.13
	29 *Sacch. mangini*	10.50	99 ,, ,,	5.55
	30 *Kloeckera apiculata*	4.70	100 *Sacch. mangini*	10.75
	31 *Sacch. mangini*	9.72	101 ,, ,,	9.72
	32 *Hansenula californica*	0.9	102 *Hansenula californica*	0.52
	33 ,, ,,	0.17	103 ,, ,,	0.52
	34 ,, ,,	1.12	104 *Torulopsis inconspicua*	0.54
	35 ,, ,,	0.52	105 ,, ,,	0.35
	36 *Torulopsis inconspicua*	0.72		
	37 ,, ,,	1.44		
	38 ,, ,,	1.12		

ment performed with 1 g sample has given evidence of the presence of yeasts in 50% of the cases and with 4 g of soil these microorganisms were found to be present in each soil examined.

So in half of the soils examined the number of yeasts was almost nil, not even reaching one cell per g. On the other hand also with 4 g of soil it has been observed that there is slow development of yeasts and slow formation of gas in the bottles. In fact in samples 11 and 12 fermentations became manifest after 5 days, in samples 1, 2, 4 and 8 after 6 days and in samples 3, 5, 6, 7, 9 and 10 only after 7 days. So it can be concluded, that the yeast content in all soils analysed has been decidedly low.

As to the number of species found in the various samples, in 11, taken near an apple tree, the richest results were obtained, *viz.*, 6 species. 4 species have been isolated from samples 1 and 12, 3 from sample 5 and 2 from 2, 3, 4, 6, 7, 8 and 10. From sample 9 merely 1 species of yeast was isolated.

In any case these results, while confirming what is already known, *i.e.*, that more yeasts are found near fruit trees, do not give any

TABLE 2.

Species isolated	Enrichment with 1 g		Enrichment with 4 g		From both enrichments		
	Number of positive samples	Number of strains isolated	Number of positive samples	Number of strains isolated	Frequency of positive samples		Number of strains isolated
					Number	%	
Non-sporeforming species	5	16	11	41	11	91.7	57
1. *Kloeckeria apiculata*	2	6	11	18	11	91.7	24
2. *Torulopsis inconspicua*	1	3	5	14	5	41.5	17
3. *Torulopsis pulcherrima*	–	–	1	4	1	8.3	4
4. *Candida Guilliermondii*	1	2	1	2	2	16.6	4
5. *Rhodotorula mucilaginosa*	1	1	1	1	1	8.3	2
6. *Aureobasidium pullularia*	3	4	2	2	3	24.9	6
Sporeforming species	5	22	8	26	8	66.5	48
7. *Sacch. ellipsoideus*	–	–	1	4	1	8.3	4
8. *Sacch. mangini*	2	7	2	4	2	16.6	11
9. *Hansenula saturnus*	3	6	5	14	5	41.5	20
10. *Hansenula californica*	1	4	1	2	1	8.3	6
11. *Pichia membranefaciens*	1	5	1	2	1	8.3	5
Totals	6	37	12	67	12	100	105

definite evidence that manuring has any influence on the flora of *Saccharomycetes* in the soil. In fact from the greater part of the differently manured soils two species have been isolated, as it also was the case for soil 6 which was kept unmanured.

In table 2 the results are reported in such a way that species isolated, divided into spore-forming and non-sporeforming types are listed along with the number of samples in which they are present and with their frequency of occurrence. Totals for non-sporeformers, for spore-formers and the total number of positive samples for each separate species are inserted.

In total 11 species of yeast have been found, 6 of which non-sporeformers and 5 sporeformers. *Kloeckeria apiculata* was the most prevalent, being present in 11 soils (91.7%). *Hansenula saturnus* and *Torulopsis inconspicua* were found in 5 soils (41.5%), whilst *Aureobasidium pullulans* (*Dematium pullulans*) has been found in 3 soils (24.9%) and *Candida Guilliermondii* in 2 soils (16.6%) whereas *Torulopsis pulcherrima*, *Saccharomyces ellipsoideus*, *Saccharomyces mangini*, *Rhodotorula mucilaginosa*, *Hansenula californica* and *Pichia membranefaciens* were present in only 1.

Non-sporeforming yeasts appear to be more widespread. They were found in 11 soils (91.7%), whilst the frequency of sporeforming yeasts amounts to 66.5% of the soils examined.

Summary.

In 12 Netherlands soil samples, in two successive analyses carried out in the autumn of 1952, the author has isolated up to 105 cultures of yeasts to be classified under the following species: *Kloeckera apiculata*, *Torulopsis inconspicua*, *Torulopsis pulcherrima*, *Candida Guilliermondii*, *Rhodotorula mucilaginosa*, *Aureobasidium pullulans*, *Hansenula saturnus*, *Hansenula californica*, *Saccharomyces mangini*, *Saccharomyces ellipsoideus* and *Pichia membranefaciens*.

In all the soils examined the quantity of yeasts found has been very small. The results obtained do not offer evidence for any relationship between manurial treatment and species and number of yeasts occurring. Sporeforming yeasts were less numerous than non-sporeforming ones.

Riassunto.

Da 12 terreni Olandesi, in due successive analisi, eseguite nell'autunno 1952, l'A. riferisce di avere isolato ben 105 colture pure di lieviti da riportare alle seguenti specie: *Kloeckera apiculata, Torulopsis inconspicua, Torulopsis pulcherrima, Candida Guilliermondii, Rhodotorula mucilaginosa, Aureobasidium pullulans, Hansenula saturnus, Hansenula californica, Saccharomyces mangini, Saccharomyces ellipsoideus* e *Pichia membranefaciens.*

In tutti i terreni esaminati la quantità di lieviti riscontrati è stata molto esigua; non è stata posta in evidenza alcuna influenza in rapporto alle diverse concimazioni ed è stata rilvata la scarsissima diffusione delle specie sporigene su quelle incapaci di formare spore.

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(From the National Institute of Public Health, Utrecht).

A COMPLEMENT FIXING TOXOPLASMA ANTIGEN PREPARED FROM DUCK EGGS AND ENHANCED BY PHENOL

by

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(Received September 9, 1954).

Several methods for the preparation of complement fixing Toxoplasma antigens from infected fluids or tissues have been described. Steen and Kåss (1951) obtained an antigen from ascitic fluid of infected mice. They inoculated the mice with Toxoplasma intraperitoneally. After 4—5 days the ascitic fluid was collected. The fluid was centrifuged and the sediment was taken up in the original volume of saline to make a 10% suspension. The suspension was repeatedly freezed and thawed in order to free the antigen.

Westphal (1952) prepared a similar antigen from infected guinea-pigs, whereas Gaillard (1952) obtained antigens from infected rabbit lungs.

Warren and Russ (1948) described a method for preparing an antigen from infected chorioallantois of chicken embryos. We chose this method for further investigation. An ascitic fluid antigen was taken as a standard. It appeared, however, that egg antigens were less potent, unless the antigen was freed from the cells as much as possible. Good results were gained by the following procedure.

The chorioallantois of 11—12 days old duck embryos is infected with a Toxoplasma strain, which is adapted to propagation in eggs. The membranes are inoculated with 0.1 ml of a 1% suspension of infected chorioallantois in broth, pH 7.2, which contains 400 U of penicilline and 100 mg streptomycine per ml. The eggs are incubated at 38°C. for 6 days, after which membranes with heavy reactions are selected for antigen production. The membranes are weighed, minced with scissors in a mortar and homogenized to a 10% suspension in 0.85% sterile saline. The suspension is centrifuged at a rate of 3000 r.p.m. for half an hour. The sediment is resuspended in saline to the original volume. This washing is repeated twice. The sediment of the last washing is resuspended in 0.85% saline which contains 0.5% phenol and 0.05% sodium azide. This suspension is homogenized in a Waring blender for 4 minutes and is then frozen in a deep-freezer at —25°C. Next day the frozen material is thawed slowly at room temperature. The freezing-thawing process is repeated once on each of two successive days. After the last thawing, the suspension is centrifuged at a rate of 2000 r.p.m. The clear supernatant can be stored at —25°C. and used as an antigen.

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Various conservatives, such as phenol, merthiolate and sodium azide were tried and compared as to the effect on antigenic reactivity. It is known that raw antigens of lymphogranuloma venereum can be potentialized considerably by heating or phenolizing (Nigg *et al.*, 1946). The immunogenic potency of rabies vaccine can be enhanced by adding merthiolate (Nelson and Anderson, 1953). Our toxoplasma antigen derived from eggs could be potentialized by adding phenol in a concentration of 0.5%. The addition of merthiolate and sodium azide or heating were useless in this respect.

Comparative complement fixation tests were carried out with the two antigens made from infected chorioallantois and ascitic fluid. Serum from rabbits which were immunized against purified antigen from mice was used. This serum reacted specifically with toxoplasma antigen and showed no reaction with normal tissue antigens from duck embryos and only a slight reaction with normal mouse antigens. Fixation of complement was performed at +6° C during 18 hours. Complement and amboceptor were used in constant quantities of 2 M.H.D. each.

The results are presented in table 1. If a slight reaction with normal mouse antigens in the ascitic fluid preparations is taken into account, it can be admitted that the egg antigen reacts as good as the ascitic fluid antigen. The reactivity of the two antigens is clearly enhanced by the addition of phenol and sodium azide. Though fluids are generally more suitable for antigen purification than tissues, preference is given to the egg membrane antigen as described above, because the latter can be obtained more easily in large quantities for routine work.

Summary.

A method for the preparation of Toxoplasma antigen from infected duck chorioallantois is described. The reactivity of toxoplasma antigens can be enhanced by adding phenol.

TABLE 1.

Results of complement fixation tests with different toxoplasma antigens [1]).

Dilution of immune rabbit serum No. 618	Dilutions of Toxoplasma antigen made from infected chorioallantois																Dilutions of normal chorioallantois antigen with 0.5% phenol and 0.05% sodium azide		
	without conservatives								with 0.5% phenol and 0.05% sodium azide										
	1	2	4	8	16	32	64	128	1	2	4	8	16	32	64	128	1	2	4
4	4	4	4	3	1	±	—	—	4	4	4	4	3	2	1	1	—	—	—
8	4	4	2	1	—	—	—	—	4	4	4	3	2	2	±	—	—	—	—
16	4	4	2	—	—	—	—	—	4	4	3	2	1	1	—	—	—	—	—
32	4	3	1	—	—	—	—	—	4	4	2	1	±	—	—	—	—	—	—
64	3	1	±	—	—	—	—	—	4	3	1	—	—	—	—	—	—	—	—
128	1	±	—	—	—	—	—	—	3	2	1	—	—	—	—	—	—	—	—
256	—	—	—	—	—	—	—	—	2	±	—	—	—	—	—	—	—	—	—
512	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

	Dilutions of Toxoplasma antigen made from infected ascitic fluid																Dilutions of normal mouse antigen with 0.5% phenol and 0.05% sodium azide		
	without conservatives								with 0.5% phenol and 0.05% sodium azide										
	1	2	4	8	16	32	64	128	1	2	4	8	16	32	64	128	1	2	4
4	4	4	4	4	3	2	1	—	4	4	4	4	4	3	2	1	1	±	—
8	4	4	4	3	1	—	—	—	4	4	4	4	3	1	1	—	±	—	—
16	4	4	2	1	—	—	—	—	4	4	4	4	2	1	±	—	—	—	—
32	4	3	1	±	—	—	—	—	4	4	4	2	1	—	—	—	—	—	—
64	3	2	±	—	—	—	—	—	4	3	2	1	—	—	—	—	—	—	—
128	1	1	—	—	—	—	—	—	4	2	1	—	—	—	—	—	—	—	—
256	—	—	—	—	—	—	—	—	3	1	±	—	—	—	—	—	—	—	—
512	—	—	—	—	—	—	—	—	1	±	—	—	—	—	—	—	—	—	—

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[1]) 4 = ++++; 3 = +++; 2 = ++; 1 = +.

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EIN NEUER ASCOMYCET AUS AFRIKA

von

J. A. VON ARX

(Eingegangen am 4. Juni 1954).

Das Centraalbureau voor Schimmelcultures in Baarn übergab mir in dankenswerter Weise drei Agarkulturen eines Ascomyceten. Zwei davon stammten von Prof. Dr W. J. LÜTJEHARMS in Bloemfontein, der eine Kultur aus Erde von Rietvlei in der Nähe von Pretoria isoliert, eine andere aus Johannesburg von Dr KRUPKO erhalten hatte. Die andere Kultur isolierte Herr H. SWART aus Erde von Frankenwald bei Johannesburg. Eine vierte, im Kongogebiet aus Erde isolierte Kultur des Pilzes sandte mir Herr J. MEYER in Brüssel.

Bei diesem Ascomyceten handelt es sich um einen interessanten, zu den *Unitunicatae* sensu LUTTRELL (1951) zu stellenden Pyrenomyceten, der im Baue der Perithecien und der zugehörigen Konidienform gut zur Gattung *Neocosmospora* E. F. Smith passt und sich von *N. vasinfecta,* der einzigen Art der Gattung, nur durch die Sporen unterscheidet. Diese besitzen beim vorliegenden Pilz ein aussen glattes Epispor von überall ungefähr derselben Dicke, während dieses bei *N. vasinfecta* unregelmässige Verdickungen und eine rillenförmige Ziselierung aufweist (MOREAU, 1950).

Trotz diesen abweichenden Sporenmerkmalen muss der vorliegende Pilz als zweite Art zu *Neocosmospora* gestellt werden:

Neocosmospora africana spec. nov.

Mycelium ex hyphis copiose et dense ramosis, septatis, hyalinis vel subhyalinis, 2.5—4 μ crassis compositum. Perithecia greges irregulares formantia vel dispersa, globulosa vel ovata, 200—280 μ lata, 260—400 μ alta, ad verticem in collo late cylindraceo vel doliformo, 80—110 μ diam., poro periphysiter lato pertusum transeuntia. Pariete carnoso, 18—28 μ crasso, e stratis cellularum irregulariter angulatarum 15—25 μ diam. metientium, pellucide lateritarum vel aurantiacarum composito, intus subito in stratum hyalinum indistincte concentrice fibrosum transeunte.

Asci numerosi, cylindracei, antice obtuse rotundati, vix vel leniter, postice plerumque paullo magis attenuati et in stipitem brevem transeuntes, tenuiter unitunicati, 8-spori, 90—110 × 9—12 μ.

Sporae monostichae, late-ellipsoideae vel globosae, utrinque rotundatae, continuae, primo hyalinae, postea pellucide flavo-brunneae, 9—12 × 7—10 μ. Paraphyses late filiformes, ex cellulis hyalinis 15 μ diam. metientibus, mucosae.

Hab. in vitro ex terra Africae australis, Johannesburg, Frankenwald, leg. W. J. LÜTJEHARMS.

Der Pilz bildet ein die Agaroberfläche überziehendes, je nach dem Nährboden verschieden stark ausgebildetes, weisses oder hellgraues Luftmycelium. Dieses besteht aus 2.5—4 μ dicken, sich oft verzweigenden, septierten, hyalinen Hyphen. Die herdenweise oder dicht zerstreut dem Mycel aufsitzenden Perithecien sind kugelig-flaschenförmig, hell, ziegelrot oder bräunlich, 200—280 μ breit und die zylindrisch-kegelige oder kugelig aufgezetzte Mündung eingerechnet, 260—400 μ hoch. Die fleischig-häutige Wand besteht aus einigen Lagen von eckigen, flachen, fast plattenförmigen, 15—25 μ grossen Zellen mit ungefähr 2 μ dicken, rötlichen Wänden (Abb. 1). Die 80—110 μ hohe und ebenso breite, von einem innen reich mit fädigen, ca. 2 μ dicken, bis 20 μ langen, hyalinen Periphysen besetzten Kanal durchbohrte Mündung besteht aus rundlich-eckigen, dünnwandigen, 10—14 μ grossen Zellen, von denen die äusseren oft halbkugelig vorgewölbt sind. Zwischen dem eigentlichen Gehäuse und der aufgesetzten Mündung befindet sich oft eine Einschnürung.

Die ziemlich zahlreich parallel stehenden Asci (Abb. 1) entspringen einer das Gehäuseinnere bekleidenden, aus flachen, hyalinen Zellen bestehenden Schicht. Sie sind zylindrisch, oben breit abgerundet, oft etwas verjüngt und dann gestutzt, unten in einen kurzen, ziemlich breiten Stiel verschmälert, enthalten acht einreihig gelagerte Sporen, besitzen eine zarte, einfache Membran und messen 90—100 × 9—12 μ. Die Sporen (Abb. 2) sind breit ellipsoidisch oder fast kugelig, jung hyalin, reif durchscheinend hellbraun, 9—12 × 7—10 μ gross und besitzen ein ca. 0.5 μ dickes, aussen völlig glattes, innen oft etwas unregelmässiges Epispor. Keimporen oder Keimspalten fehlen. Zwischen den Asci befinden sich paraphysenartige, breite, aus sehr dünnwandigen, gestreckten, bauchigen, hyalinen, früh verschleimenden, bis 20 μ grossen Zellen bestehende Fäden, dagegen fehlen eigentliche Paraphysen.

Die nur spärlich akrogen in kleinen Köpfchen oder einzeln entstehenden Konidien sind länglich, ellipsoidisch oder fast spindelförmig, gerade oder gebogen, ein-, selten zweizellig, hyalin und 3—12 × 2—4 μ gross.

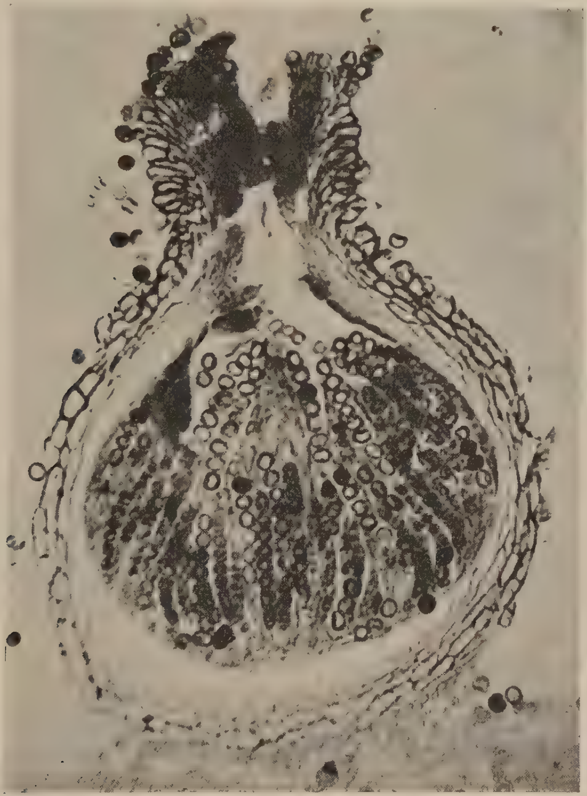

Abb. 1. Medianschnitt durch ein Perithecium von *Neocosmospora africana.* Vergr. 275 mal. Nach einem mit Haematoxylin gefärbten Mikrotomschnitt.

Die Gattung *Neocosmospora* gehört zu den *Sphaeriales* und muss zu den *Sordariaceae—Xylariaceae* gestellt werden (vergleiche VON ARX und MÜLLER, 1954). Ihre Diagnose ist, was die Sporenmerkmale betrifft, in dem Sinne zu erweitern, dass sie auch Formen mit einem glatten Epispor enthalten kann.

Mit den *Nectriaceae* hat der vorliegende Pilz nur die Farbe der Gehäuse gemeinsam und unterscheidet sich von der amerosporen Gattung *Pseudonectria* Seaver (Syn.: *Nectriella* Sacc. non Nits., *Notarisiella* (Sacc.) Clem. et Shear) durch die gefärbten, breit ellipsoidischen, nicht spindelförmigen Sporen, sowie durch die Wachstumsweise (vgl. PETCH, 1938; VON ARX und MÜLLER, 1954).

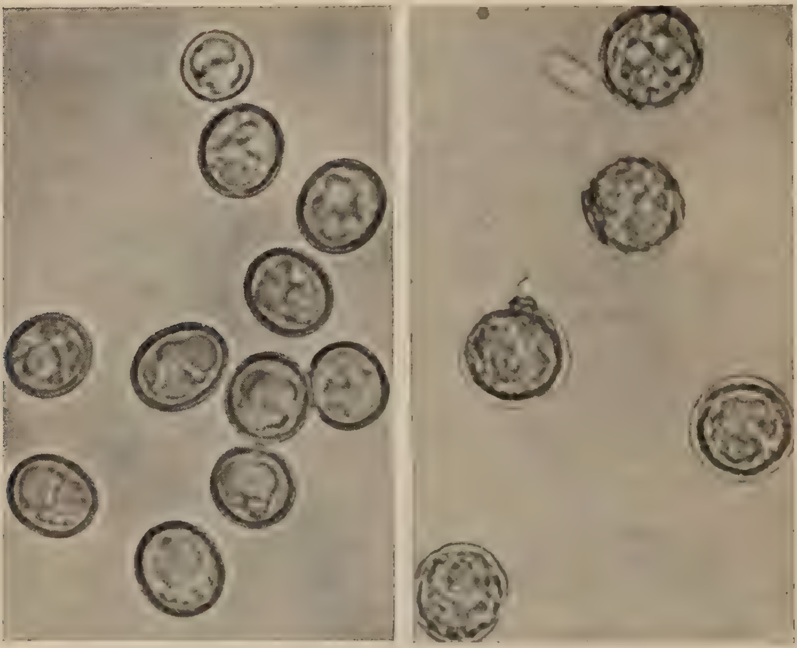

Abb. 2. Ascosporen, links von *Neocosmospora africana*, rechts von *N. vasinfecta*. Vergr. 1200 mal.

Sordaria Ces. et de Not. im Sinne von CAIN (1934) und die damit näher verwandten Gattungen sind durch die dunkle Farbe der Gehäuse oder wenigstens der Mündungen verschieden; ferner zeichnen sie sich durch die Sporen aus, die Schleimhüllen, Anhängsel, Keimporen oder bei *Coniochaeta* Sacc. Keimspalten besitzen und meist auch dunkler gefärbt sind. Die Gattungen *Neurospora* Shear et Dodge und *Gelasinospora* Dowd. haben wohl oft helle Gehäuse; die Sporen besitzen aber ein mit Skulpturen, Leisten oder Höckern versehenes, im Alter dunkles Epispor.

Melanospora Cda. (Syn.: *Sphaeroderma* Fuck.) in der Umgrenzung, die VON ARX und MÜLLER (1954) der Gattung geben, hat wohl glatte Sporen, unterscheidet sich aber von *Neocosmospora* durch die keuligen oder fast kugeligen, sehr vergänglichen Asci, durch die Sporen, die Keimporen oder Keimspalten besitzen, ferner durch die gewöhnlich mit Wimperhaaren gekrönte, oft verlängerte Mündung.

Die kürzlich von Petrak (1952) beschriebene Gattung *Gaeumanniella* unterscheidet sich durch hyaline Sporen, sowie durch die in senkrechter Richtung gestreckten, keuligen, unten gestielten, einem krustenförmigen Subiculum aufsitzenden, sehr dünnwandigen Perithecien. Sie scheint eine isolierte Stellung einzunehmen.

Die als amerospore *Nectriaceae* beschriebene Gattung *Mycaureola* Maire et Chemin endlich muss gestrichen werden. Bei den von den Autoren als einzellig und kugelig beschriebenen Sporen handelt es sich ohne Zweifel um Oeltröpfchen in den noch unentwickelten Schläuchen.

Neocosmospora africana wie auch *N. vasinfecta* zeigen vor allem im Bau der Fruchtschicht eine gewisse Uebereinstimmung mit den typischen Vertretern der Gattung *Physalospora* Niessl und sind wie diese in phylogenetischer Hinsicht sehr interessante Pilze, die innerhalb der *Sphaeriales* auf einer noch tiefen Stufe stehen und verwandtschaftliche Beziehungen zu den *Nectriaceae*, den *Melanosporaceae* und den *Sordariaceae-Xylariaceae* zeigen.

Frl. G. M. T. Cramer hat von *Neocosmospora africana* Mikrotomschnitte angefertigt, wofür ich ihr auch hier meinen Dank aussprechen möchte.

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CLADOSPORIUM AVELLANEUM DE VRIES, A SYNONYM OF „*HORMODENDRUM*" *RESINAE* LINDAU

by

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In 1952 a new species has been described in the genus *Cladosporium* as *Cladosporium avellaneum* de Vries (1952). The fungus has been isolated from "Nivea" ointment at Utrecht, 5—1947.

By monospore isolation four different saltants have been selected which for more than three years have been carried unchanged. Two of these saltants produced morphologically different fruiting structures. A paniculate conidial structure is produced by the forma *viride,* whereas a globular to conical fruiting structure is a character of the forma *avellaneum.* The forma *albidum* is morphologically identical to the forma *viride* differing only by its lack of pigment; the forma *sterile* is a pigmented and entirely sterile saltant.

In July 1954 the author checked two strains of the so-called creosote fungus, *Hormodendrum resinae* Lindau and immediately recognized the striking resemblance of the Enola strain of this fungus with *Cladosporium avellaneum.* MARSDEN (1954) who studied this strain, also noted two distinct morphologic strains differing in the shape of their fruiting structures. His New York isolate strain agrees with *Cladosporium avellaneum* forma *avellaneum,* his New York saltant strain with the forma *viride.*

Twelve years before, CHRISTENSEN, KAUFERT, SCHMITZ and ALLISON (1942) gave an account of a similar strain which they provisionally called *Hormodendrum resinae* Lindau. The strain used by CHRISTENSEN a.o. differs from the Enola strain and from *Cladosporium avellaneum* by its production of tiny black sclerotia in the agar medium. MARSDEN mentioned a difference in spore colour, the isolate of CHRISTENSEN having olivegreen spores, whereas the Enola isolate shows brown spores. The strains studied by MARSDEN and

CHRISTENSEN a.o. are very tolerant to high concentrations of creosote and coal tar. It is this peculiar biological character that has been carefully studied by the two last-mentioned authors.

As none of the American authors checked the type material, the first thing to do was to examine LINDAU's type of *Hormodendrum resinae*. This specimen appeared to be in the herbarium of the Botanical Garden and Museum at Berlin-Dahlem and has been described in RABENHORST's Kryptogamen-Flora VIII, 1907, p. 699.

The examination of the type revealed that it was not distinct from *Cladosporium avellaneum* forma *viride* de Vries.

As the strains studied by MARSDEN and CHRISTENSEN a.o. were able to grow on a basal mineral agar medium containing either 4% coal tar or 1% creosote, an experiment has been performed in which five strains: 1. Enola, 2. "Christensen" (received from MARSDEN), 3. *Cladosporium avellaneum* forma *avellaneum*, 4. *Cladosporium avellaneum* forma *viride* and 5. *Cladosporium avellaneum* forma *albidum* were cultured on the above-mentioned media. The basal mineral medium and the method were the same as that used by MARSDEN (1954, p. 615).

Contrary to expectation the last three strains did not grown on these media, while the Enola and Christensen isolates made a good growth.

This failure to grow, however, does not exclude the identity of the Enola strain with *Cladosporium avellaneum*, as it is a common character in mycology that there is a considerable inter-strain variability of physiological properties. Although further experiments could not be performed, it is likely that the three *Cladosporium avellaneum* strains are able to grow in lower concentrations of creosote or coal tar. Perhaps they can even be gradually accustomed to higher concentrations.

The study of the morphology of these fungi has revealed that *Hormodendrum resinae* Lindau and *Cladosporium avellaneum* de Vries forma *viride* de Vries are identical. The name *Hormodendrum resinae* Lindau, therefore, has priority and *Cladosporium avellaneum* de Vries is a synonym of it. As *Hormodendrum* Bonorden is a doubtful genus, it became necessary to make this new combination: *Cladosporium resinae* (Lindau) nov. comb., forma *resinae*, Syn.: *Cladosporium avellaneum* de Vries forma *viride* de Vries.

The names for the other three forms, *avellaneum, albidum* and *sterile,* can be retained.

Summary.

1. *Cladosporium avellaneum* de Vries forma *viride* de Vries is a synonym of *"Hormodendrum" resinae* Lindau.
2. *Hormodendrum resinae* Lindau has been transferred to the genus *Cladosporium*. The new combination is *Cladosporium resinae* (Lindau) nov. comb. forma *resinae*.
3. The names of the three saltants, forma *avellaneum*, forma *albidum* and forma *sterile* can be retained.
4. After the first inoculation from malt agar to these media it was impossible to grow the strains originally described as *Cladosporium avellaneum* de Vries on a basal mineral medium containing either 1% creosote or 4% coal tar.

The author wishes to express his gratitude to the Director of the Botanical Gardens and Museum at Berlin-Dahlem for his kindness in providing him with the original type material.

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(Laboratory of Bacteriology and Hygiene of Domestic Animals,
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INVESTIGATIONS ON SOME PROPERTIES OF *LISTERELLA MONOCYTOGENES*

by

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(Received September 25, 1954).

Listerella monocytogenes was first isolated in England by Murray, Webb and Swann (1926) during an outbreak of disease in laboratory rabbits and guinea pigs. It is known that the term "*Listerella*" has been previously used for protozoa; Pirie (1940) proposed the name "*Listeria*", but the latter is also a homonym. The terms "*Listerella*" and "Listerellosis" seem to be firmly established and will continue to be generally used (Jordan and Burrows, 1945).

Most of the authors mention *L. monocytogenes* as the only representative of the genus, nevertheless Hauduroy (1937) describes *L. bessoni*, that was discovered by Besson (1924) in normal and pathological human faeces; the latter was previously designated by Besson as *Vibrio iners* and in any case it does not have the same importance as the former.

In Bergey's manual the following characteristics are mentioned for *L. monocytogenes*: small rods, 0.4 to 0.5 by 0.5 to 2.2 μ, with rounded ends, slightly curved in some culture media; occur singly or in V-shaped or parallel pairs and in short chains; motile, peritrichous, with four flagella at ordinary temperatures, with tendancy toward non-motility or single flagellum at 37°C.; not acid-fast, gram-positive.

Topley and Wilson remark that they resemble in many aspects members of the erysipelothrix-group, non sporing, non capsulated, facultative anaerobic. The following bacteriological properties are generally admitted:

agar: dew drop like culture
gelatin: no liquefaction
broth: slightly turbid
liver broth: turbid, luxurient culture
litmus milk: no clotting, slightly acid, reduction in the lowest part of the tube (8 hours)
neutral red agar: unchanged
Gersbach: no indol production
hydrogen sulfide: negative
methyl red: positive
Voges-Proskauer: negative
catalase: positive
blood agar: β hemolysis
filtrate: non toxic.

Our studies concerned 4 strains of *L. monocytogenes*: 2 isolated from cattle (H and D) and 2 isolated from poultry (C and M). These 4 strains showed all the above mentioned characteristics. For identification, culture in litmus milk is the most suitable. The same strains were tested on their biochemical behaviour; the obtained results are given in Table 1.

TABLE 1.

	L	H	D	C	M	
Arabinose	—	(—)	±	+	+	L = literature
Rhamnose	+	+	—	+	(+)	H = bovine strain
Xylose	+	+	—	+	—	
Dextrose	+	+	+	(—)	(—)	D = bovine strain
Galactose	—	—	—	(—)	(—)	
Lactose	(+)	—	—	—	—	C = avian strain
Maltose	(+)	(+)	(+)	(+)	—	
Sucrose	(+)	(+)	+	—	—	M = avian strain
Trehalose	+	+	+	+	(—)	
Raffinose	—	—	—	—	—	
Melicitose		—	—	—	—	+ = positive
Inulin	—	—	—	—	—	— = negative
Dextrin	—	(+)	±	—	—	(+) = slowly positive
Salicin	+	+	+	—	—	
Dulcitol	—	—	—	+	+	(—) = negative after having been positive
Inositol	—	—	—	—	—	
Glycerol	+	(+)	—	(+)	—	
Mannitol	—	—	—	—	—	
Sorbitol	—	—	—	—	—	± = dubious
Esculin		+	+	+	+	

Fermentation always takes place without gas production. All the cultures were observed during 30 days; in some instances positive reaction occurred very slowly; in other ones a positive reaction was noted at the beginning, but afterwards the latter became negative. In a few cases reaction was questionable.

HARVEY and FABER believed that a correlation would exist between the fermentation of melicitose and some serological types; in our investigations neither bovine, nor avian strains fermented this sugar.

Antigenic analysis of *L. monocytogenes* was done by several workers. We immunized rabbits by means of cultures of strain H on dextrose broth and made precipitation tests with extracts of our 4 strains prepared according to the method of FULLER. The highest titres were obtained with the homologous strain H (bovine) and the avian strain C and a lower titre for the strains M as well as D. Most of the authors claim that no differentiation may be obtained by means of this test and that the constitutive polysaccharide is common to all strains.

Agglutination tests are generally used for differentiation. It appears from studies of PATERSON (1939), JULIANELLE (1940), SAVIGNY and VILLAZON (1941), ROBINS and GRIFFIN (1945) that besides common H- and O-antigens, there will exist some other ones owing to an existing difference in types. Unfortunately the number of types established on this basis differ from one author to another. Excepting for a few strains (human NYFELDT, rabbit MURRAY), no correlation could be found between the antigenic types, host specificity and geographical distribution. HIRATO *et al.* have found all known serological types in a single species (sheep). We tried differentiation by O-, H- and full-agglutination. In a first series we made use of bloodserum from rabbits injected with live cultures of 3 strains (H, C and M). Results are given in Table 2.

TABLE 2.

	O			H			F		
	H	C	M	H	C	M	H	C	M
Ser. H	**1/80**	—	—	**1/80**	—	—	**1/160**	—	—
Ser. C	1/20	**1/320**	1/160	1/20	**1/320**	1/160	—	**1/640**	1/160
Ser. M	1/20	1/20	**1/320**	1/20	1/80	**1/160**	—	1/80	**1/320**

In a second series (table 3) a fourth strain (D = bovine) was available. For the strains C and M we used the serum of the same rabbits as in the first series, but fresh ones had to be prepared for the strains D and H (rabbit of first series died).

TABLE 3.

	O				H				F			
	H	C	M	D	H	C	M	D	H	C	M	D
Ser. H	**1/80**	1/40	1/160	1/80	**1/640**	1/320	1/160	1/320	**1/160**	1/160	1/40	1/80
Ser. C	1/80	**1/80**	—	1/20	1/80	**1/80**	—	1/20	1/80	**1/80**	1/20	1/20
Ser. M	1/80	1/320	**1/320**	1/20	1/320	1/320	**1/640**	—	1/80	1/80	**1/160**	1/40
Ser. D	1/160	1/320	1/160	**1/160**	1/320	1/640	1/320	**1/640**	1/80	1/160	1/80	**1/320**

As may be stated, homologous sera did not always give agglutination at the highest dilution and no marked differences were noted between O-, H- and F-agglutination.

L. monocytogenes may be isolated easily from visceral lesions, but not from nervous localizations. At first isolation was done by streaking suspect nervous substance on blood agar plates for the detecting of β-hemolytic colonies. Later on enrichment methods were applied, keeping nervous substance for a long time in glycerol or in broth in the refrigerator. With the latter method we succeeded in isolating *L. monocytogenes* from suspect brain substance after a period of 4 weeks. SHIMIZU *et al.* (1945) have mentioned that enrichment may be obtained after 48 hours by adding guanofuracin at a concentration of 1/10.000 to broth, but we had no opportunity to try this.

Besides bacteriological and serological tests for identification of *L. monocytogenes* attention may be called to two biological methods. The first consists in provoking experimentally monocytosis in rabbits or guinea pigs. When infection was not followed by a fatal issue, we could state that in rabbits monocytosis reached a maximum after approximately 6 days, decreasing slowly till a normal rate at about 5 weeks. After reinfection at that moment, monocytosis increased again, without reaching the first top level. A third infection at the 55th day had no result. In fig. 1, influence of injections on the other blood cells is shown; note the opposite (quite symmetric) effect in polynuclears and lymphocytes.

The second method is the eye-reaction. As mentioned in literature, the application of a few drops of a young culture of *L. monocytogenes* on the conjunctiva of a rabbit provokes a specific reaction, consisting in an acute conjunctivitis, sometimes accompanied by keratitis and by a profuse production of exsudate. No other microorganisms would be able to give the same reaction. This method was unsuccessful, until we made previous scarifications on the conjunctiva. From that moment we recorded always typical reactions, whenever suspect cultures were containing *L. monocytogenes*. As a rule the inflammatory process regresses after one week, without generalization. The average cellular content of the exsudate is given in table 4.

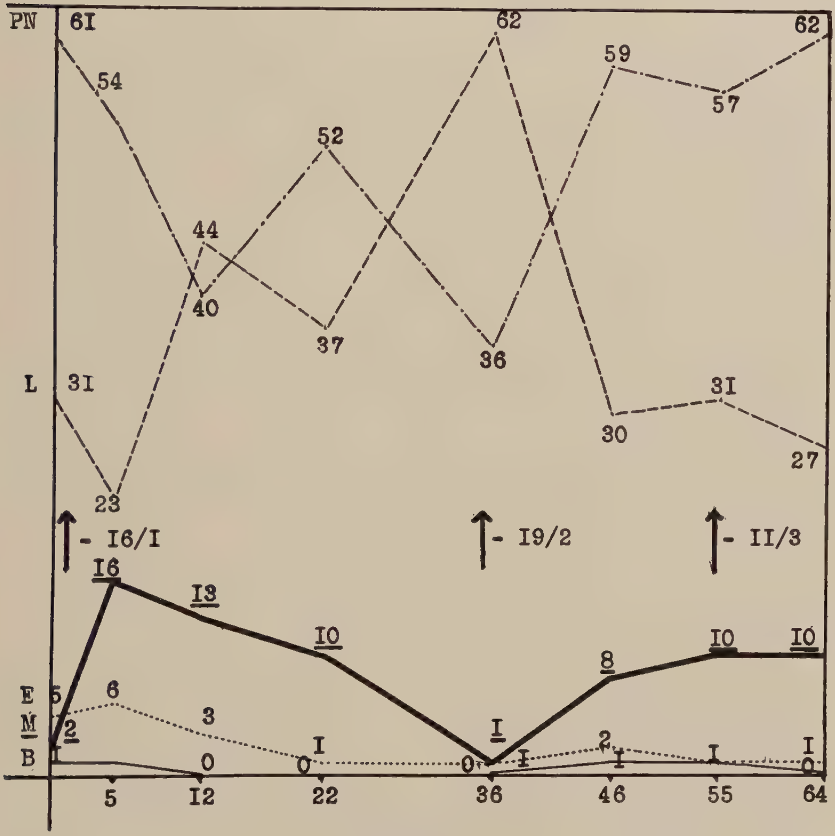

Fig. 1. PN = polynuclears neutrophiles; L = lymphocytes; E = eosinophiles; M = monocytes; B = basophiles.

TABLE 4.

Neutrophiles (segm. nucl.)	21
id. (nonsegm. nucl.)	5
Eosinophiles	51
Basophiles	—
Monocytes	20
Lymphocytes	3

Although in accordance with other authors, we never succeeded in cultivating *L. monocytogenes* from this exsudate, nevertheless after a few days antibodies could be shown in the bloodserum.

Experimental receptivity of laboratory rodents along different routes is generally admitted. Intravenously injected rabbits died after 1 or 2 days from septicaemia, without specific lesions; by other modes (conjunctiva, subcutis, brain) we did not always succeed in provoking a fatal disease, but latent infection could be demonstrated by positive culture from the blood and by agglutination. From 4 guinea pigs infected subcutaneously, 3 died after 3—6 days, showing at post mortem examination numerous foci in liver and spleen. All attempts for infecting laboratory rats were vain. The most suitable rodents were mice; they always died after approximately 5 days with typical lesions at autopsy.

Adult poultry seems to be less receptive; even after intravenous infection fatal issue occurs seldom. On the contrary young chicks are very sensitive and die after a short period with specific lesions. As mentioned in the literature, pigeons would be non receptive; in our experiments it was an exception for adult pigeons to die after infection, but generally they remained carriers for a long period. Probably receptivity of pigeons is the same as in poultry, but young pigeons are never used for experimental purposes. The most susceptible animals are small birds (sparrows, canaries, finches, etc.); they always die after approximately 6 days, with typical necrotic foci in the internal organs.

Finally we studied the action in vitro of substances, bacteriostatic against *L. monocytogenes*. As will be seen in table 5, results are not always in accordance with those recorded in practice.

Differences may be observed among various sulphonamides; at a concentration of 0.01% only sulphanilamide, sulphamezathin and marbadal will inhibit growth and from the antibiotics only penicillin and aureomycin. Attention will be drawn to the fact that these bacteriostatics had no influence on the vitality of *L. monocytogenes*,

TABLE 5.

Sulfonamides (%)	1	0.1	0.01	C	T
Sulphanilamide (UCB)	—	—	—	+	+
Marprontil (Bayer)	—	—	+	+	+
Ladogal (Böhring)	—	—	+	+	+
Sulphamezathin (ICI)	—	—	—	+	+
Marbadal (Bayer)	—	—	—	+	+
Sulphadiazin	—	—	+	+	+
Antibiotics (units)	**100**	**10**	**1**	**C**	**T**
Penicillin	—	—	—	+	+
Bacitracin	—	—	+	+	+
Aureomycin	—	—	—	+	+
Dihydrostreptomycin	—	—	+	+	+
Terramycin	—	—	+	+	+
Chloromycetin	—	—	+	+	+

C = check without bacteriostatics; T = culture from inhibiting medium on normal one.

for cultures made from the inhibiting medium on a ordinary one gave always a normal growth.

Résumé.

Après un court aperçu historique les auteurs font part de quelques recherches effectuées au sujet des propriétés culturales et biochimiques de *L. monocytogenes*. Du point de vue antigénique aucune différentiation n'a pu être obtenue au moyen de la précipitation, quant à l'agglutination, celle-ci a donné des résultats assez divergents. L'isolement à partir de substance nerveuse a pu être opéré après enrichissement par séjour prolongé à la glacière. L'isolement au moyen de milieux furacinés n'a pu être tenté. Il est fait part de quelques résultats obtenus lors de monocytose expérimentale chez le lapin. La réaction oculaire a donné des résultats fidèles, si au préalable la conjonctive avait été scarifiée. Parmi les animaux de laboratoire, les souris et les petits passereaux constituent les réactifs de choix. Le lapin et le cobaye donnent des résultats irréguliers, alors que la volaille et le pigeon adultes deviennent en général des infectés latents. Quelques sulfamidés, de même que la pénicilline et l'auréomycine inhibent à doses réduites la croissance de *L. monocytogenes*.

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ETUDE DU PROCESSUS DE TRANSFORMATION DES ACIDES DI- ET TRICARBOXYLIQUES CHEZ *PENICILLIUM BREVI-COMPACTUM*

par

PAUL GODIN [1])

(Reçu le 31 Août 1954).

INTRODUCTION.

L'analyse chromatographique du mycélium (GODIN, 1954*a*) et du milieu de culture (GODIN, 1953) de *P. brevi-compactum* (souche Db182A) a permis de déceler, parmi les produits du métabolisme du glucose, les acides citrique, α-cétoglutarique, succinique, fumarique, malique et oxalacétique. La mise en évidence de ces corps pose le problème de l'existence du cycle de KREBS ou d'un processus analogue chez cette moisissure.

Malgré les travaux de différents auteurs dont NORD et WEISS (1951) ont récemment donné une vue d'ensemble, le processus de transformation des acides di- et tricarboxyliques chez les moisissures n'est pas encore parfaitement connu. Cette note décrit les premières recherches entreprises en vue de résoudre ce problème chez *P. brevi-compactum.*

MÉTHODES EXPÉRIMENTALES.

Conditions de culture.

Le *P. brevi-compactum* étudié ici est la souche Db182A de la mycothèque de BIOURGE (Laboratoire de Microbiologie de l'Université de Louvain).

La moisissure est ensemencée dans des erlenmeyers de 500 ml de capacité, sur 200 ml de milieu Czapek-Dox. Ce milieu de culture est en effet celui qui favorise le plus la production des acides di- et tricarboxyliques par la moisissure. Après développement complet du microbe à 25° C., on substitue au milieu de culture initial, 100 ml d'une des diverses solutions expérimentées.

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Méthodes d'analyse.

Le dosage du glucose de la solution substituée est réalisé par polarimétrie. La consommation des acides organiques des solutions substituées est suivie par titrimétrie ou par mesure du pH de la solution de métabolisme.

On recherche les acides di- et tricarboxyliques produits par la moisissure, par la chromatographie sur papier. La solution de métabolisme est acidifiée par H_2SO_4, puis extraite à l'éther sulfurique en trois opérations successives, chaque fois par le double de son volume d'éther. La solution d'extraction est concentrée à petit volume par distillation et soumise à l'analyse chromatographique suivant les méthodes décrites dans des publications antérieures (Godin, 1953—1954 *a*).

LES RÉSULTATS EXPÉRIMENTAUX.

Influence de l'acide pyruvique sur le métabolisme des acides citrique, succinique et malique.

La solution substituée, renfermant 2% d'un des acides citrique, d,l-malique ou succinique, est additionnée ou non d'acide pyruvique à l'une des doses croissantes que voici: 0.25%, 0.50% et 0.75%. Le pH de cette solution est amené à la valeur 4.0 par l'addition de la quantité voulue de soude.

La consommation des acides par la moisissure se traduit par une hausse du pH de la solution de métabolisme, hausse d'autant plus rapide que le métabolisme est plus intense. Le tableau 1 donne la valeur du pH des différentes solutions après 6 jours d'incubation.

On voit qu'à une concentration égale ou inférieure à 0.5%, l'acide pyruvique n'a guère d'effet sur l'intensité du métabolisme des acides citrique et d,l-malique, mais accélère par contre la consommation de l'acide succinique. Une teneur en acide pyruvique égale à 0.75% ralentit le métabolisme, tout particulièrement celui de l'acide citrique.

L'analyse chromatographique des diverses solutions de méta-

TABLEAU 1.

pH des solutions d'acide citrique, d,l-malique ou succinique additionnées ou non d'acide pyruvique, après 6 jours d'incubation.

Teneur initiale en acide pyruvique	pH des solutions renfermant l'acide citrique	pH des solutions renfermant l'acide de d,l-malique	pH des solutions renfermant l'acide de succinique
nulle	7.75	8.4	5.5
0.25%	7.35	8.5	7.5
0.50%	7.6	8.4	7.5
0.75%	5.75	8.25	6.4

bolisme permet de déceler, outre les corps préexistants et les substances phénoliques produites par la moisissure, les composés suivants:

— **l'acide malique** dans la solution d'acide citrique dépourvue d'acide pyruvique et, à l'état de trace, dans la solution d'acide succinique additionnée ou non d'acide pyruvique;

— **l'acide α-cétoglutarique** dans les solutions d'acide citrique ou d'acide malique additionnées d'acide pyruvique;

— **l'acide citrique** dans les solutions d'acide malique ou d'acide succinique additionnées ou non d'acide pyruvique;

— **l'acide fumarique** dans les solutions d'acide succinique ou d'acide malique additionnée ou non d'acide pyruvique.

Influence de l'acide acétique sur le métabolisme des acides citrique et succinique.

La solution substituée, renfermant 2% d'acide citrique ou d'acide succinique, est additionnée ou non d'acide acétique à une des concentrations croissantes que voici: 0.1, 0.2, 0.3 ou 0.4%. Cette solution est amenée à pH 4.0 par addition de la quantité voulue de soude.

La consommation des acides s'accompagne d'une hausse du pH d'autant plus rapide que le métabolisme est plus intense. Le tableau 2 donne la valeur du pH des solutions de métabolisme après 6 jours d'incubation.

L'acide acétique ralentit d'autant plus le métabolisme de l'acide succinique que sa concentration est plus élevée, ce qui n'était pas le cas avec l'acide pyruvique. Par contre, à concentration inférieure ou égale à 0.2%, il accélère le métabolisme de l'acide citrique, tandis qu'il le ralentit si sa teneur atteint ou dépasse 0.3%.

TABLEAU 2.

pH des solutions d'acide succinique ou citrique additonnées ou non d'acide acétique, après 6 jours d'incubation.

Teneur initiale en acide acétique	pH des solutions renfermant l'acide succinique	pH des solutions renfermant l'acide citrique
nulle	7.3	6.3
0.1%	7.3	8.1
0.2%	6.8	7.7
0.3%	5.75	5.8
0.4%	4.6	5.0

L'analyse chromatographique des solutions de métabolisme permet de déceler, outre les corps préexistants et les substances phénoliques produites par la moisissure, les composés suivants:

— **l'acide citrique** dans les solutions d'acide succinique additionnées ou non d'acide acétique;

— **l'acide malique** dans les solutions d'acide citrique additionnées ou non d'acide acétique et, à l'état de trace, dans les solutions d'acide succinique additionnées ou non d'acide acétique;

— **l'acide fumarique** dans les solutions d'acide succinique additionnées ou non d'acide acétique;

— **l'acide succinique** dans les solutions d'acide citrique renfermant initialement 0.3% ou plus d'acide acétique.

Influence de l'acide malonique sur le métabolisme.

L'étude de l'influence de cet inhibiteur de la succinodéshydrogénase sur le métabolisme de la moisissure a été réalisée en substituant au milieu de culture initial, d'une part, une solution à 3% d'acide succinique ou d'acide citrique, renfermant 1% d'acide malonique mi-neutralisé par $NaHCO_3$, et, d'autre part, une solution à 5% de glucose additionnée de la même quantité de malonate acide de sodium. Rappelons que la moisissure est capable de dégrader l'acide malonique (Godin, 1955*a*).

La présence d'acide malonique à cette concentration ne modifie pas la vitesse de consommation du glucose qui a disparu après 11 jours d'incubation, ni celle de l'acide succinique qui est totalement consommé après 8 jours d'incubation. Par contre, l'acide malonique ralentit la consommation de l'acide citrique qui, pour être totale, nécessite 14 jours d'incubation, alors qu'en l'absence d'acide malonique, elle est réalisée en 8 jours.

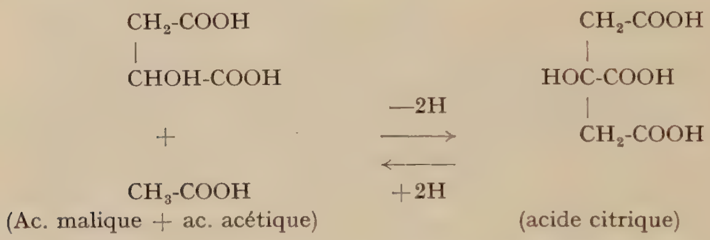
L'analyse chromatographique de la solution glucosée permet de mettre en évidence, outre les corps préexistants et les substances phénoliques produites par la moisissure, les acides **malique, citrique, succinique** et **fumarique.** Par contre, dans les solutions renfermant l'acide succinique ou l'acide citrique, on ne trouve, comme substances produites par la moisissure, que les composés phénoliques.

Discussion.

On a vu, dans une note antérieure (Godin, 1955*a*), que la moisissure qui dégrade facilement les acides citrique, α-cétoglutarique, succinique, fumarique, d,l-malique, pyruvique et acétique, est incapable de consommer l'acide isocitrique, un des intermédiaires du cycle de Krebs. On a, d'autre part, signalé dans cette même publication que, chez la moisissure originaire du milieu Czapek-Dox, le rendement moléculaire de la transformation biochimique de l'acide succinique en substances phénoliques s'élève à 0.024, tandis que le rendement obtenu avec l'acide citrique n'atteint que la valeur 0.0164. Si le processus de dégradation des acides di- et tricarboxyliques chez *P. brevi-compactum* suivait le cycle de Krebs parcouru dans son sens normal, une molécule d'acide citrique devrait nécessairement donner naissance à une molécule d'acide succinique. Dans ce cas, le rendement moléculaire de la transformation de l'acide citrique en substances phénoliques devrait atteindre une valeur au moins égale à celle obtenue avec l'acide succinique. On peut donc déduire de ces faits que le processus de transformation des acides di- et tricarboxyliques chez *P. brevi-compactum* n'est pas identique au cycle de Krebs classique.

Les travaux relatés ici, rapprochés des résultats obtenus antérieurement, permettent toutefois de se faire une idée vraisemblablement assez exacte du processus en question chez *P. brevi-compactum.*

Ainsi, il semble exister chez la moisissure une relation biochimique particulièrement étroite entre l'acide citrique et l'acide malique. On constate en effet que si l'acide citrique est décelé parmi les produits du métabolisme, il est toujours accompagné de l'acide malique. Ces deux acides sont notamment décelés dans le milieu Czapek-Dox des cultures agitées et des cultures en surface (Godin, 1953) et dans le mycélium de ces mêmes cultures (Godin, 1954). On les trouve tous deux parmi les produits du métabolisme de l'acide succinique, tandis que l'acide malique est un produit du métabolisme de l'acide citrique et, inversément, l'acide citrique un produit du métabolisme de l'acide malique. Il y a donc tout lieu de croire que la synthèse de l'acide citrique se réalise à partir de l'acide malique, très probablement suivant le schéma proposé par Chrzaszcz et Tiukow (1930) et représenté dans la figure 1. On notera que, si l'acide acétique n'a jamais été décelé parmi les produits du métabolisme de la moisissure, on a, par contre, trouvé son précurseur immédiat, l'acétaldéhyde (Godin, 1955*b*).



$$\begin{array}{lcl} CH_2\text{-}COOH & & CH_2\text{-}COOH \\ | & & | \\ CHOH\text{-}COOH & & HOC\text{-}COOH \\ & -2H & | \\ + & \xrightarrow{\hspace{1cm}} & CH_2\text{-}COOH \\ & \xleftarrow{\hspace{1cm}} & \\ CH_3\text{-}COOH & +2H & \end{array}$$

(Ac. malique + ac. acétique) (acide citrique)

Fig. 1. Schéma proposé de la synthèse de l'acide citrique chez *P. brevicompactum.*

Là où on décèle l'acide malique, il arrive cependant qu'on ne mette pas en évidence l'acide citrique. Cela se constate dans le seul cas où la solution de glucose substituée au milieu Czapek-Dox des cultures agitées ou des cultures en surface, est additionnée de carbonate de calcium (Godin, 1953—1954*b*). Ceci peut s'expliquer en admettant que $CaCO_3$ inhibe, directement ou indirectement, la formation de l'acide citrique à partir de l'acide malique et de l'acide acétique.

Si, dans la solution glucosée additionnée de $CaCO_3$, on ne décèle pas l'acide citrique, on trouve par contre, en compagnie de l'acide malique, les acides succinique, fumarique et oxalacétique, ce dernier seulement dans le cas des cultures en surface (Godin, 1953—1954*b*). Entre ces dernières substances, il semble exister une relation biochimique étroite. On trouve en effet les acides fumarique et malique dans la solution de métabolisme de l'acide succinique et l'acide fumarique dans celle de l'acide malique. D'autre part, si, parmi les produits du métabolisme du glucose, on décèle l'acide fumarique, on met également en évidence les acides succinique et malique. Tel est le cas dans le milieu Czapek-Dox des cultures agitées (Godin, 1953) dans le mycélium des cultures agitées et des cultures en surface sur le même milieu (Godin, 1954*a*) et, comme il vient d'être dit, dans la solution substituée de glucose additionnée de $CaCO_3$ (Godin, 1953—1954*b*). Il semble donc très vraisemblable que le processus représenté dans la figure 2, dont l'existence chez les moisissures a d'abord été supposée par Chrzaszcz et Tiukow (1930), se déroule chez *P. brevi-compactum.*

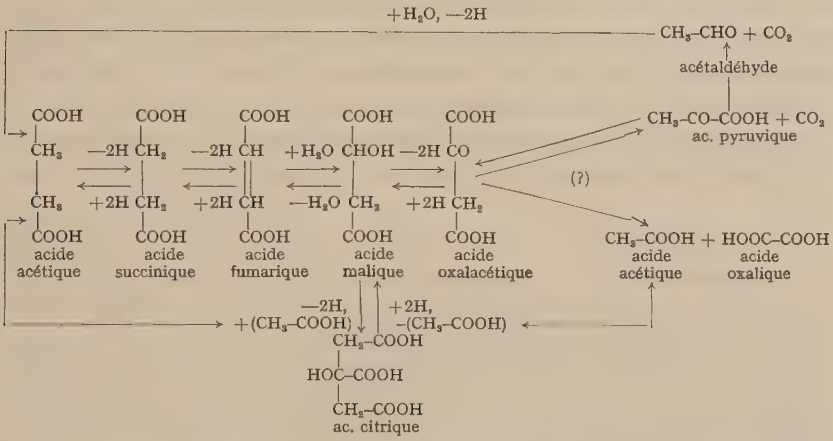


Fig. 2. Schéma proposé des transformations des acides di- et tricarboxyliques chez *P. brevi-compactum.*

D'autres arguments peuvent être invoqués en faveur de cette hypothèse. Rappelons les raisons exposées au début de cette discussion, qui ont fait supposer que le cycle de Krebs ne se déroule pas chez *P. brevi-compactum,* raisons qui militent plus ou moins directement en faveur du schéma proposé. On peut y ajouter le fait que l'acide succinique n'est pas trouvé dans les produits du métabolisme de l'acide citrique, sauf si on ajoute à la solution substituée au moins 0.3% d'acide acétique, alors que, inversément, on trouve toujours l'acide citrique parmi les produits du métabolisme de l'acide succinique. Rappelons aussi que l'addition d'acide malonique à la solution substituée d'acide citrique ne provoque aucune accumulation d'acide succinique dans la solution de métabolisme.

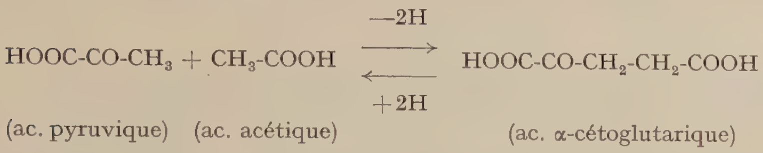
Tels sont donc les motifs qui font supposer que le processus suggéré correspond bien à la réalité. Les résultats d'une des expériences décrites dans cette note semblent toutefois infirmer l'hypothèse avancée. Il s'agit de la mise en évidence, à côté de l'acide succinique, des acides citrique, fumarique et malique dans la solution de glucose additionnée d'acide malonique, tous corps qui ne sont pas décelés dans la solution glucosée dépourvue de cet inhibiteur. En effet, si, chez *P. brevi-compactum*, les acides organiques subissent les transformations admises, on comprend qu'on puisse déceler l'acide succinique dans la solution de métabolisme du glucose, en présence d'un inhibiteur de la succinodéshydrogénase, mais on s'explique difficilement à priori que, dans ces conditions, les acides fumarique, malique et citrique s'accumulent en quantité plus grande qu'en l'absence de cet inhibiteur. Notons d'ailleurs que si le processus de transformation de ces acides était identique au cycle de Krebs, il serait tout aussi étonnant de constater dans les mêmes conditions, une certaine accumulation des acides fumarique et malique.

Pour expliquer cette "anomalie", tout en admettant le schéma de transformation des acides di- et tricarboxyliques proposé, on peut supposer que les acides citrique, fumarique et malique sont formés par suite de l'inversion du processus admis. Cette inversion serait due à l'accumulation des produits de la dégradation du glucose, notamment les acides acétique et pyruvique, par suite de l'inhibition par l'acide malonique d'une des étapes de la voie normale de transformation de ces corps.

Une autre explication à cette "anomalie" suppose que le schéma proposé est incomplet. Un des acides dicarboxyliques, autre évidemment que l'acide succinique, pourrait être synthétisé par la moisissure, par une voie différente de celle qui est suggérée ici. C'est le corps en question qui serait à l'origine des autres acides décelés sauf, évidemment, l'acide succinique.

Quant à l'acide α-cétoglutarique, c'est un produit du métabolisme qui est rarement décelé. On le trouve dans le milieu Czapek-Dox des cultures agitées (Godin, 1953) et dans la solution de métabolisme des acides citrique et malique, mais uniquement dans le cas où de l'acide pyruvique a été ajouté dans la solution de substitution. Tenant compte de ce dernier fait, on peut supposer que l'acide α-cétoglutarique dérive des acides pyruvique et acétique par la réaction représentée dans la figure 3.

Cette réaction de synthèse de l'acide α-cétoglutarique est vraisemblable, car on vient de voir que le cycle de Krebs semble bien ne pas se dérouler chez la moisissure. D'ailleurs, l'addition d'acide malonique à la solution substituée de glucose ou d'acide citrique



$$HOOC\text{-}CO\text{-}CH_3 + CH_3\text{-}COOH \underset{+2H}{\overset{-2H}{\rightleftarrows}} HOOC\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}COOH$$

(ac. pyruvique) (ac. acétique) (ac. α-cétoglutarique)

Fig. 3. Schéma proposé de la synthèse de l'acide α-cétoglutarique chez *P. brevi-compactum.*

ne provoque nullement la mise en évidence d'acide α-cétoglutarique, ce à quoi on aurait pu s'attendre si le cycle de Krebs existait chez la moisissure. L'hypothèse avancée ici est également corroborée par une remarque de Simonart et Chow (1954), lesquels constatent que l'acide α-cétoglutarique n'est décelé parmi les produits du métabolisme d'*Aspergillus oryzae* que si on ajoute de l'acide pyruvique à la solution nutritive.

Résumé.

L'étude du processus de transformation des acides di- et tricarboxyliques chez *P. brevi-compactum* a permis d'établir que le cycle de Krebs ne se déroule pas chez cette moisissure. Les résultats des recherches menées jusqu'à ce jour font au contraire supposer, avec grande vraisemblance, que le processus étudié est identique au schéma proposé par Chrzaszcz et Tiukow. Quant à l'acide α-cétoglutarique, il semble qu'il soit synthétisé par condensation oxydative d'une molécule d'acide pyruvique et d'une molécule d'acide acétique.

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(From the National Institute of Public Health, Utrecht).

THE FLOCCULATION OF TETANUS TOXIN AND TOXOID. II.

by

J. D. VAN RAMSHORST and **H. RIJKS**

(Received September 2, 1954).

In a previous paper (van Ramshorst and Rijks, 1954) we discussed the various flocculation zones which can occur in tetanus toxins and toxoids obtained from a modified Tarozzi broth, at the same time comparing the values we had found with the A.B.V. (antitoxin-binding value determined in vivo) and the M.T.V. (maximal toxin value, i.e. the maximal quantity of actual toxin, as ascertained by means of tests in which mixtures of toxin and antitoxin are directly injected into mice).

For details of these techniques we may refer to the paper in question. We have now been able to get further confirmation of the results then published by means of more extensive experiments, which have also revealed some interesting new facts.

FLOCCULATION-VALUES ETC. IN THE COURSE OF AN INCUBATION-PERIOD OF THIRTY DAYS.

In the above publication we described an experiment in which tetanus cultures were incubated for 12 days, during which period they were analysed every day. This time the contents of two flasks were harvested and analysed every second day and this was done 15 times (over a total period of 30 days, therefore).

Table 1 shows the results of this experiment. In addition to the 4 flocculations found before, there appeared to be a 5th, "lower" than the other four. This lowest flocculation had so far remained unnoticed, owing to the fact that it requires a much longer flocculation-time (Kf) than the others (2—5 hours). As the Lf-value of this flocculation always is very low, it can only be discerned with certainty after a fairly long period of incubation (more than 12

TABLE 1.

Analysis of tetanus toxin during 30 days' incubation.

Days	D.l.m. (peptone)	Lf (upper line) and Kf (lower line) in hours					M.T.V.	A.B.V.
2	1 : 20.000	38	28	10	5?		0.125	0.536
		9	14½	14½	?			
4	1 : 40.000	64	25	17	3		>0.425	3.00
		4½	5½	3	5½			
6	1 : 70.000	132	32	19	4½		1.50	4.00
		4½	2	2	4			
8	1 : 390.000	170	44	22	6		2.25	5.50
		2	2½	2½	3			
10	1 : 180.000	212	86	48	11		3.37	11.00
		3¼	¾	¾	1¾			
12	1 : 300.000	220	94	58	12½		3.75	13.75
		2¾	¾	¾	1¾			
14	1 : 190.000	180	92	68	12½	4½	3.75	14.85
		⅓	¼	¼	1¼	3		
16	1 : 180.000	144	108	96	17	4½	4.00	14.85
		1¼	⅓	¾	¾	2½		
18	1 : 100.000	100	112	88	18	5	3,75	17.50
		½	½	¾	1	2½		
20	1 : 80.000	112	76		18	4	4.00	19.00
		½	½		¾	2¾		
22	1 : 80.000	116	98	48	18	4	4.38	14.30
		½	½	¾	1¼	2¼		
24	1 : 80.000	100	124	40	16½	4	3.75	14.85
		½	¼	1	1	2½		
26	1 : 40.000	92	108	20	16	3	2.50	11.00
		½	¼	1	1	?		
28	1 : 40.000	92	108	16	8		2.60	6.90
		½	¼	¾	1½			
30	1 : 20.000	70		16		2?	1.05	3.75
		1½		2¾		?		

days; afterwards we found that this flocculation is also regularly to be seen in cultures of about 10 days, if one knows what to look for and waits long enough). In fig. 1 this flocculation is indicated by curve A.

Above this curve A is the B curve; i.e. the curve of the flocculation which we used to regard as the "lowest flocculation" and which was found to agree well with the A.B.V. determined by the above-mentioned in vivo test. This was also found to be the case in the present experiment (cf. fig. 2). This Lf increases until about the 18th or 20th day, after which it decreases fairly quickly. The same applies to the A.B.V.

Let us now look at the three higher curves. The highest curve, E, goes down fairly quickly after 10—12 days, which was also the case in our previous experiments. Between the 16th and the 20th day these three flocculations are about equally high and consequently hard to tell apart. After this we see three flocculations again (curves F, G and H), but at present it cannot be said for certain how the curves F, G and H link up with C, D and E. We only have certain indications (in the nature of the flocculi), that G might be the continuation of E. For the rest the situation inside the circle in fig. 1 is uncertain.

A.B.V., M.T.V. AND LOWER FLOCCULATIONS.

Curves A and B of fig. 1 are shown on a different scale in fig. 2, in which A.B.V. and M.T.V. have also been inserted. In addition to the B curve coinciding with the A.B.V. curve, as already stated, we now see that the M.T.V. rises for about 12 days and then remains more or less constant for another 10 days, after which there is a falling off. Flocculation curve A practically coincides with this M.T.V. curve.

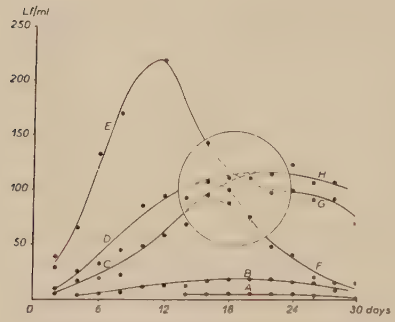

Fig. 1. Flocculation in tetanus toxin during 30 days' incubation.

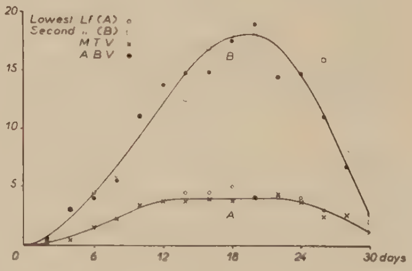

Fig. 2. The two lowest flocculations compared with M.T.V. and A.B.V. during 30 days' incubation of tetanus toxoid.

Our previous assumption that the M.T.V. would always be the half of the A.B.V. was evidently wrong. After a longer period of incubation, in any case, this no longer holds good, the A.B.V. then being 4 or 5 times as high as the M.T.V. From this it follows that in all probability these two quantities are determined by two different components of the crude toxin, both binding antitoxin, but only one of them being toxic.

We are now confronted with the question whether the non-toxic component can act as an antigen and stimulate the production of antitoxin. In order to obtain data on this matter we made the following experiment:

A crude tetanus toxin, the lowest flocculation values (A and B curves) of which were 4 and 9 Lf/ml respectively (M.T.V. 4.5; A.B.V. 8.25), was concentrated by ultrafiltration. After the process these values (determined after dilution of final product to original volume) were: 3 and 9 Lf/ml; M.T.V. 3; A.B.V. 8.25. As the toxin had been concentrated to 1/8 of its original volume, these values in the undiluted ultrafiltration residuum were therefore: Lf 8 × 3 = 24 and 8 × 9 = 72; M.T.V. 8 × 3 = 24; A.B.V. 8 × 8.25 = 66. To 250 ml of this residuum we added 1.21 ml of tetanus antitoxin of 5400 A.U./ml, *i.e.* 6530 A.U. The 250 ml of toxin with M.T. V. 24 contained 250 × 24 = 6000 toxin units, in terms of M.T.V. Therefore the mixture contained an over-measure of antitoxin of almost 10%, which was meant to ensure that the antitoxin would be sufficient to bind all the toxin (M.T.V.).

The mixture was now stood in a water bath at 45° C. until about $\frac{3}{4}$ hour after a flocculation had become clearly visible. The flocculi were then removed by means of a centrifuge ¹). The supernatant was now found to flocculate normally at 19 Lf/ml, the A.B.V. being 22. As was to be expected the M.T.V. was 0, and mice, injected with the undiluted supernatant did not show any trace of tetanus symptoms.

Consequently the toxic component had been quantitatively removed and part of the non-toxic antitoxin-binding component was still present. Naturally, part of the latter had also been removed, in fact about 50% more than can be theoretically calculated, but this is by no means surprising, as it is common knowledge that such flocculation precipitates adsorb more antigen from an over-measure than they should do according to theoretical equivalence.

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¹) Previous experiments (VAN RAMSHORST and RIJKS, 1954, p. 25) had already shown, that toxin proper is not affected by standing at 45°C., followed by centrifuging.

In any case, there was enough of it left (19 Lf/ml, A.B.V. 22). Some of this was diluted to 10 Lf/ml (checked by flocculation, and some was adsorbed on aluminium phosphate (5 mg $AlPO_4$/ml, 10 Lf/ml). Guinea-pigs were then "immunized" with these.

For comparison a crude tetanus toxoid was concentrated to 48 Lf/ml by ultrafiltration and part of this was also diluted to 10 Lf/ml and another part adsorbed on aluminium phosphate (5 mg $AlPO_4$/ml, 10 Lf/ml). Guinea-pigs were immunized with these too.

Further particulars of these injections and the results are shown in table 2. Blood samples of all the guinea-pigs had been taken previous to the first injection in order to exclude the possibility of the test animals having a measurable immunity before the experiment. They were all found to have a serum titer < 0.01 A.U./ml.

TABLE 2.

(For explanation see text).

Fluid supernatant of toxin	Fluid toxoid		Supernatant of toxin adsorbed on $AlPO_4$		Toxoid adsorbed on $AlPO_4$	
9 guinea pigs 3 × 1 ml at intervals of 4 weeks	9 guinea pigs 3 × 1 ml at intervals of 4 weeks		14 guinea pigs 2 × 0.5 ml at intervals of 4 weeks		13 guinea pigs 2 × 0.5 ml at intervals of 4 weeks	
Serum titers 4 weeks after last injection	Serum titers 4 weeks after last injection		Serum titers 4 weeks after last injection		Serum titers 4 weeks after last injection	
All animals <0.01 A.E.	0.03	2.0	<0.01	<0.1	2.0	12.0
	0.3	0.03	0.01	<0.01	2.0	1.5
	0.02		0.03	<0.01	7.0	0.5
	2.0		<0.01	0.2	0.5	1.0
	0.2		<0.01	0.3	4.5	1.0
	0.02		<0.01		0.75	5.0
	0.05		<0.01		1.0	
Mean <0.001	Mean 0.5		Mean 0.05		Mean 3.0	

After the immunization, by means of 3 injections of 1.0 ml of the fluids and 2 injections of 0.5 ml of the adsorbed substances, the difference between the effect of normal toxoid and the supernatant portion of the toxin proved to be very great. The antitoxin titers obtained with toxoid were on an average at least 50 times as high as those obtained with the toxin supernatant. In fact, the latter can hardly be said to have had any effect at all.

DISCUSSION.

We may draw the conclusion that *Clostridium tetani* produces several substances, two of which bind the specific tetanus antitoxin. One of these two is toxic (spasmine), the other is not. These two substances are probably produced independently. The non-toxic component might be regarded as a "spontaneous toxoid", but there is nothing to indicate that the non-toxic component could arise from the toxic element. If the change toxin → "spontaneous toxoid" was so rapid as would have to be the case, if this were true, one could never expect a reasonably stable tetanus toxin.

Moreover, the above experiments have shown that this non-toxic component has little, if any, immunizing effect and this is another reason why one cannot very well speak of a "toxoid". Only few guinea-pigs showed a slight reaction, but this may very well have been caused by some very slight vestiges of the toxin-antitoxin flocculi, which can cause an immunization reaction in a very sensitive guinea-pig [1]. Although, therefore, some very slight effect is not definitely ruled out by the above experiments, it can only have been very inconsiderable, the antitoxin titers being 50 × as low as those obtained with normal formol-toxoid. This will be seen if one compares the titers obtained after immunization both with the fluids and with the substances adsorbed on aluminium phosphate.

As a practical consequence of this, the "normal" flocculation value (curve B, corresponding with the antitoxin-binding) is in fact no measure of the immunizing element. The long-known fact, that the immunizing effect of tetanus toxoids does not depend exclusively on the flocculation value, may also be partly due to this, although in this case there are other factors, too (degree of purity, partial denaturing of the toxoid protein with the retention of Lf, etc.).

Summary.

1. In the flocculation reaction of tetanus toxins prepared in a modified Tarozzi broth there appear five flocculation zones.

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[1] When guinea-pigs are immunized with tetanus-prophylactics, some of the animals usually show extremely high titers, far above the average.

2. In addition to the "lowest flocculation" described in our previous paper, which corresponds with the antitoxin-binding (A.B.V.), we found another, still lower, flocculation, the value of which is about the same as the so-called maximal toxin value (M.T.V.).
3. A.B.V. was found to be from 2 to 5 times as high as M.T.V.
4. During the period of incubation all flocculation values show an initial rise, which is followed by a fall after 10—20 days.
5. *Clostridium tetani* produces a toxic and a non-toxic element, both binding antitoxin, but the latter having little, if any, immunizing effect.

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ON THE PATHOGENESIS OF DIPHTHERIA

IV. ON THE OCCURRENCE OF DIPHTHERIA TOXIN IN THE SALIVA OF DIPHTHERIA PATIENTS [1]

by

A. TASMAN, J. E. MINKENHOF, A. C. BRANDWIJK and **L. SMITH**

(Received September 8, 1954).

In a recent publication Tasman and Smith (1954) showed that under certain circumstances fresh human saliva is a suitable medium for *C. diphtheriae* and that this microbe can sometimes produce fairly large quantities of diphtheria toxin in this saliva.

When fresh, some of the saliva samples that were examined proved to have a marked inhibitory effect on the production of this toxin. This inhibition must probably be ascribed to bacterial antagonism, more especially of streptococci versus diphtheria bacteria (Bibby, Hayne and Clough, 1938; Mühlenbach, 1939; Thompson and Shibuya, 1946; Belding and Belding, 1948). The agent responsible for this inhibition is very labile. Not only is it rendered completely inactive by autoclaving, but the inhibiting effect is also considerably reduced by keeping the saliva at room-temperature, or at 34—35° C., for a short time. Even when these measures were not taken, however, diphtheria toxin was found in nearly all the examined saliva samples after inoculation with a *C. diphtheriae* culture and incubation at 34—35° C. The well-known P.W.-8 strain as well as fresh strains isolated from patients were used for this purpose.

Although the saliva-samples, which were all obtained from healthy persons, who did not carry diphtheria bacteria were found to differ greatly with respect to their inhibitory effect on the formation of toxin, it was a remarkable fact, that saliva-samples from the same persons usually showed the same mutual differences in the various experiments.

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[1] Part I, II and III: Antonie van Leeuwenhoek **19**, 135, 333, 1953; **20**, 417, 1954.

In view of the limited material, however, it was not possible to ascertain the existence of certain saliva-types in this respect. These experiments also showed that much depends on the nature of the diphtheria strain which is used.

When we considered the pathological conditions of the diphtheria patient in the light of all these facts, it occurred to us that it must be possible under certain favourable conditions to demonstrate the presence of diphtheria toxin in the saliva of diphtheria patients. This problem is the subject of the present study. Saliva collected from 50 patients after the diagnosis of diphtheria had been bacteriologically confirmed was tested for diphtheria toxin.

From adults and children over four years old the saliva sample was obtained in a very simple manner, *viz.*, by making them spit about 1 ml of saliva down a glass funnel into a fitting glass tube. In the case of small children who had difficulty in spitting, the saliva was collected by carefully swabbing the oral cavity with a plug of sterile cotton-wool, care being taken to keep well away from the back of the throat and especially to avoid contact with patches of membrane if these were present. The saturated tampon was then put in a glass tube containing 1.5 ml of saline, into which the saliva was thus diffused. We also tried to prevent the patients from taking any food or drink shortly before the saliva was collected, lest the saliva which had been present in the mouth for some time (more or less in a "state of rest") should be replaced by "newly-produced" saliva. Unfortunately, this measure was not always possible. The obtained saliva-samples were always prepared for use with the least possible delay.

The saliva produced in the form of spittle was centrifuged at 3000 r.p.m. for 15 minutes. The clear fluid thus obtained was diluted with two parts of saline and sterilized by passing it through a membrane-filter ¹). The saliva obtained by means of a tampon

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¹) "Tietz Apparat", obtainable from the Membranfiltergesellschaft, Sartorius Werke A.G. & Co., Göttingen, the "Membranfilter Mittel" being used as filter.

in saline was filtered in the same way without previous centrifuging.

In order to find out whether the original saliva-samples contained any diphtheria-toxin, one and the same guinea-pig was injected intracutaneously with 0.2 ml of each of the following fluids:

1) sterile saliva-filtrate.
2) sterile saliva-filtrate mixed with diluted "normal" serum (0.3 ml saliva + 0.02 ml serum diluted 1/400 with saline).
3) sterile saliva-filtrate mixed with diluted antidiphtheria-serum (0.3 ml saliva + 0.02 ml serum diluted 1/400 with saline, i.e. 0.0005 A.U.).

The mixtures 2 and 3 both contain the same quantity of serum, but in mixture 2 (normal serum) this does not neutralize the diphtheria toxin which may be present, whereas in mixture 3 (antidiphtheria serum) it does.

4) Sterile saliva-filtrate heated at 80° C. for 20 minutes (diphtheria toxin being thermo-labile).
5) Control fluid. For this we always used 1 ml of saliva from the same healthy person, mixed with 0.12 ml of "standard toxin" diluted with saline to 1 : 1000. The saliva mixed with this quantity of diphtheria toxin was centrifuged at 3000 r.p.m. for 15 minutes, diluted with 2 parts of saline and sterilized by passing it through a membrane-filter. The 0.2 ml of this filtrate finally injected intracutaneously into the guinea-pig contained 0.000008 ml of undiluted "standard toxin". In the skin of the guinea-pig this quantity of toxin mixed with saliva-filtrate caused a red infiltrate with a diameter of about 8 mm.

The reactions caused by these injections were read after 24 and 48 hours and judged by the following criteria:

Strong positive: if the injections of 1 and 2 caused red infiltrates with a diameter of about 25 mm and the reactions to 3 and 4 could not or hardly be seen.

Positive: if the injections of 1 and 2 caused red infiltrates of the same size as or slightly larger than the infiltrate caused by the control fluid (5) and the reactions to 3 and 4 could not or hardly be seen.

Light positive: if the injections of 1 and 2 caused a red spot smaller than the one caused by the control, but larger than the reactions to 3 and 4.

Negative: if the injections of 1 and 2 caused reactions which did not differ from those to 3 and 4 and were smaller than the reaction to the control fluid (5).

The clinical course of the disease was judged and estimated by a variety of criteria. Finally, a number of strains isolated from patients were classified according to type.

Table 1 gives the results of the toxin test with patients that had not yet been injected with serum when the saliva was collected.

TABLE 1.

Results of the toxin test with saliva from patients who had not yet been injected with anti-diphtheria serum.

Patient no.	Initials of patient	Sex	Age in years	Units of anti-diphtheria-serum (AU)	Saliva or tampon	Results of the toxin test	Type of strain isolated
1	J.v.d.P.	♂	5	—	saliva	**strong positive**	—
2	M.S.	♀	5	—	,,	negative	—
3	M.C.J.	♀	5	—	,,	**positive**	—
4	C.v.L.	♂	32	—	,,	**positive**	—
5	H.I.M.S.	♂	7	—	,,	**light positive**	—
6	G.E.	♀	5	—	,,	**positive**	gravis
7	N.B.	♂	3	—	,,	negative	—
8	F.C.H.	♂	6	—	,,	**strong positive**	gravis
9	C.I.H.	♂	4	—	,,	negative	gravis
10	J.v.W.	♀	5	—	,,	**positive**	—
11	I.T.	♀	4	—	tampon	**positive**	—
12	M.	♂	2	—	,,	negative	—
13	T.S.	♀	3	—	,,	**positive**	mitis
14	C.Z.	♀	3	—	,,	**strong positive**	—
15	R.G.K.	♀	2	—	,,	**positive**	—

Of the 15 samples thus examined, 11 showed a positive reaction.

Table 2 gives the results of the toxin test with patients to whom anti-diphtheria serum had been administered just before the saliva was collected.

Of the 8 saliva samples thus examined, 5 showed a positive reaction.

Table 3 gives the results obtained with saliva-samples from patients who had been given a serum-injection one day before the saliva was collected.

Of the 17 samples thus examined, 8 showed a positive reaction.

TABLE 2.

Results of the toxin test with saliva from patients who had just been injected with anti-diphtheria-serum.

Patient no.	Initials of patient	Sex	Age in years	Units of anti-diphtheria-serum (AU)	Saliva or tampon	Results of the toxin test	Type of strain isolated
16	R.F.	♂	5	40.000	saliva	**strong positive**	—
17	G.S.	♂	2	20.000	tampon	negative	gravis
18	R.B.W.S.	♂	5	10.000	,,	negative	—
19	J.W.d.A.	♂	5	80.000	saliva	**positive**	—
20	d.H.	♂	39	60.000	,,	**light positive**	—
21	H.V.M.	♂	2	20.000	tampon	negative	gravis
22	A.T.	♀	5	20.000	saliva	**positive**	—
23	R.B.	♂	4	20.000	,,	**positive**	gravis

TABLE 3.

Results of the toxin test with saliva from patients who had been injected with anti-diphtheria-serum on the previous day.

Patient no.	Initials of patients	Sex	Age in years	Units of antidiphtheria-serum (AU)	Saliva or tampon	Results of the toxin test	Type of strain isolated
24	D.C.	♀	6	150.000	saliva	**light positive**	—
25	R.v.D.	♂	5	140.000	,,	negative	—
26	R.B.	♂	6	70.000	,,	negative	gravis
27	J.A.O.	♂	7	70.000	,,	**light positive**	—
28	Z.	♀	34	90.000	,,	negative	gravis
29	J.W.	♂	5	80.000	,,	**light positive**	—
30	J.S.	♂	3	80.000	tampon	**positive**	gravis
31	H.H.	♀	5	100.000	,,	**light positive**	—
32	E.d.H.	♂	6	40.000	saliva	**light positive**	—
33	A.V.	♀	4	50.000	tampon	negative	gravis
34	M.G.	♀	5	60.000	saliva	negative	—
35	Q.M.	♀	38	10.000	,,	negative	gravis
36	G.M.	♀	4	60.000	tampon	negative	gravis
37	J.R.	♂	6	80.000	saliva	**positive**	—
38	A.K.	♀	6	20.000	,,	negative	gravis
39	J.v.O.	♂	4	20.000	,,	negative	gravis
40	A.V.	♂	3	20.000	tampon	**light positive**	gravis

Table 4 shows the results of the toxin test with saliva-samples from patients who had had a serum-injection two days or more before the saliva was collected.

TABLE 4.

Results of the toxin test with saliva from patients who had been injected with anti-diphtheria-serum two or more days before.

Patient no.	Initials of patient	Sex	Age in years	Units of anti-diphtheria-serum (AU)	Saliva or tampon	Results of the toxin test	Type of strain isolated
41	F.M.	♀	5	80.000	tampon	negative	—
42	R.G.	♀	42	40.000	saliva	negative	gravis
43	V.H.	♀	19	180.000	,,	negative	—
44	M.v.d.P.	♀	5	240.000	,,	negative	—
45	M.S.	♀	5	190.000	,,	negative	gravis
46	N.W.	♀	4	120.000	,,	**positive**	—
47	B.T.	♂	2	60.000	tampon	negative	gravis
48	Y.v.A.	♀	6	8.000	saliva	negative	gravis

Of the 8 samples thus examined, only 3 showed a positive reaction.

Table 5 gives the results obtained with saliva-samples from 2 patients with Cutaneous and Nasal diphtheria.

TABLE 5.

Results of the toxin test with saliva from patients suffering from diphtheria of the nose and skin.

Patient no.	Initials of patient	Sex	Age in years	Units of anti-diphtheria-serum (AU)	Saliva or tampon	Results of the toxin test	Type of strain isolated
49	F.H.'	♀	5	—	saliva	negative	gravis
50	J.M.	♀	1½	30.000	tampon	negative	gravis

In neither case did we find any diphtheria toxin in the saliva.

Table 6 (p. 200) contains a number of clinical data concerning the 50 patients whom we examined.

With respect to this symptomatology we wish to point out that our choice of patient material does not give a correct picture of the age, distribution and the occurrence of mild and serious cases of diphtheria in Amsterdam in the winter of 1953—1954. It was solely determined by the fact that it was technically impossible to extend our investigation to other patients.

Table 6 gives the case histories, the symptoms and an estimate of the seriousness of the course taken by the disease. We have divided the patients into four groups according to the severity and the duration of the symptoms: +, ++, +++ and ++++ (= fatal).

What strikes one first is that the immunization of the patients still leaves much to be desired. Of the 50 patients included in our investigation, only 18 had definitely been immunized. Among the last ones was only one serious case.

Another remarkable fact is that the patients are admitted into hospital rather late and consequently are mostly injected with antitoxic diphtheria serum at a relatively far too late stage. In table 7 the patients are arranged according to the number of days elapsed between the outbreak of the disease and admission into hospital.

TABLE 7.

Patients arranged according to day of admission.

Day of disease patient was admitted	Number of patients	Percentage
1st day	5	(10%)
2nd day	9	(18%)
3rd day	16	(32%)
4th day	4	(8%)
5th day	8	(16%)
6th day	3	(6%)
7th day	5	(10%)

There is no complete parallelism between the course taken by the disease and the number of days between outbreak and admission into hospital (see table 6).

In the description of the size of the patches of false membrane this was only compared with the tonsil and mentioned, whether the false membranes had invaded the pillars of fauces and palate. We do not think it right to compare the size of the coating with the dimensions of a coin.

TABLE 6. Clinical data of

Patient no.	Initials of patient	History of diphtheria	Immunized	Day of disease on which admitted	Number of days false membrane lasted	Maximal extent of false membrane	Bull-neck	Myo-car-ditis
1	J.v.d.P.	—	?	3	6	2½ tonsil	+	+
2	M.S.	2 years before	+	3	7	2 tonsil	+	+
3	M.G.J.	—	—	5	10	2 tonsil	+	+
4	C.v.L.	—	—	3	6	2 tonsil	—	+
5	H.I.M.S.	—	—	3	6	1½ tonsil	—	—
6	G.E.	—	—	3	4	2 × ½ tonsil	—	—
7	N.B.	—	—	2	4	1½ tonsil	light +	—
8	F.C.H.	—	3 years before	3	8	half palate	+	++
9	C.I.H.	—	—	2	4	2 × ½ tonsil	—	—
10	J.v.W.	—	—	4	5	1½ tonsil	—	—
11	I.T.	—	2 years before	4	6	half palate	+	+
12	M.	—	,,	1	7	1½ tonsil	—	—
13	T.S.	—	—	2	5	1½ tonsil	—	—
14	C.Z.	—	—	3	7	2 tonsil	—	—
15	R.G.K.	—	—	1	1 d. canule	only larynx	—	—
16	R.F.	—	—	2	5	2 tonsil	—	—
17	G.S.	—	—	1	3	2 × ½ tonsil	—	—
18	R.B.W.S.	—	—	6	7	2 × ½ tonsil + larynx	—	—
19	J.W.d.A.	—	—	3	—	half palate	+	+
20	d.H.	—	—	3	5	2 × ½ tonsil	—	—
21	H.V.N.	—	—	9?	—	larynx	—	—
22	A.T.	—	1 year before	3	4	2 × ½tonsil	—	—
23	R.B.	—	—	±8	11	2½ tonsil	—	—
24	D.C.	—	?	2	3	2 × ½ tonsil	—	—
25	R.v.D.	—	—	3	6	1½ tonsil	—	—
26	R.B.	—	—	5	7	1 tonsil	—	—
27	J.A.O.	—	+	6	11	2½ tonsil	+	+
28	Z.	—	—	5	10	half palate	+	+
29	J.W.	—	—	2	4	2 × ½ tonsil	—	—
30	J.S.	—	—	5	9	half palate	—	+
31	H.H.	—	+	3	6	2½ tonsil	+	+
32	E.d.H.	—	+	1	3	2 × ½ tonsil	—	—
33	A.V.	—	—	3	6	2 × ½ tonsil	—	—
34	M.G.	—	4 years before	4	8	2½ tonsil	—	+
35	Q.M.	—	—	8	10	2 tonsil	—	—
36	G.M.	—	4 years before	3	7	1½ tonsil	+	+
37	J.R.	—	3 years before	5	7	2½ tonsil	—	—
38	A.K.	—	—	4	6	2 × ½ tonsil	—	—
39	J.v.O.	—	—	3	5	2 tonsil	+	+
40	A.V.	—	?	6	7	2 × ¼ tonsil	—	—
41	F.M.	—	—	5	—	half palate	+	+
42	R.G.	—	—	2	4	1½ tonsil	—	—
43	V.H.	—	+	3	8	2 tonsil	+	—
44	M.v.d.P.	—	4 years before	2	5	2 tonsil	—	—
45	M.S.	—	,,	5	8	2 tonsil	+	+
46	N.W.	—	14 days before	1	5	2½ tonsil	—	+
47	B.T.	—	1 month before	2	3	1 tonsil	—	—
48	Y.v.A.	—	+	5	6	2 × ½ tonsil	—	—
49	F.H.	1 year before	+	14	—	nose and skin	—	—
50	J.M.	—	—	7	—	rhinitis	—	—

tients whose saliva was tested.

ymptoms of polyneuritis	Albuminuria	Changes of the skin		Seriousness of the disease				Results of the toxin test
		knee	foot	+	++	+++	++++ fatal	
+: ey.sw.ar.ns.	+	+	+			+++		strong positive
+: sw.ns.	+	—	—		++			negative
+: ns.	—	—	—		++			positive
—	+	—	—		++			positive
—	+	—	—	+				light positive
—	—	—	—	+				positive
—	trace	—	—	+				negative
+: sw.Et.ns.nf.ar.nr.	+	+	—			+++		strong positive
—	trace	—	—	+				negative
—	—	—	—	+				positive
—	+	—	—				++++	positive
—	—	—	—	+				negative
—	trace	—	—	+				positive
—	+	—	—	+				strong positive
—	—	—	—	+				positive
—	+	—	—	+				strong positive
—	+	—	—	+				negative
+: Et.ns.	+	—	—		++			negative
+: ar.ey.	+	+	—				++++	positive
—	—	—	—	+				light positive
—	+	—	—	+				negative
—	trace	—	—	+				positive
—	+	—	—	+				positive
—	+	—	—	+				light positive
+: ns.	+	—	—		++			negative
—	+	—	—	+				negative
+: ey.ar.	—	+	—		++			light positive
+: sw.Et.ns.nf.ar.re.	+	+	—				++++	negative
—	trace	—	—	+				light positive
+: ns.ar.	+	+	+		++			positive
—	+	+	—		++			light positive
—	—	—	—	+				light positive
—	+	—	—	+				negative
+: sw.ns.	+	+	+			+++		negative
—	—	—	—	+				negative
—	—	—	—		++			negative
—	trace	+	—	+				positive
+: re.	—	—	—	+				negative
+: sw.Et.ns.ar.nr.	+	+	+			+++		negative
—	—	—	—	+				light positive
—	+	—	—				++++	negative
—	+	—	—	+				negative
—	—	—	—	+				negative
—	+	—	—	+				negative
—	+	—	—		++			negative
—	trace	+	+	+				positive
—	—	—	—	+				negative
—	—	—	—	+				negative
—	—	—	—	+				negative
—	trace	—	—	+				negative

xplanation of abbreviations:
aralysis of the eye muscles = ey
isturbances in swallowing = sw
an Ettingen symptom = Et
asal speech = ns
asal feeding = nf
– reflexia = ar
aralysis of arm, leg and respiration = re
aralysis of nervus recurrens = nr

The serious cases all had a "bull-neck", but the reverse did not always hold good. Myocarditis, if present, was always recognized from the physical symptoms and electrocardio-graphically confirmed by Dr D. Durrer (Wilhelmina Hospital). The symptoms of polyneuritis are mentioned as far as peripherally localized. It was impossible to get reliable statements concerning the power of accommodation, as this cannot be properly tested in small children. The same applies to the useful symptom of van Ettingen (1946), which we have found to be the first and last symptom of velar paralysis. For it is almost impossible to make a young child blow on the back of its hand in a sitting position (as in the usual test for herniae) in order to ascertain whether the air escapes through the nose or not.

Symmetric scaling and pigmentation with callosity on knee and instep, and occasionally along the whole front part of the lower leg were also observed in some cases, particularly in those suffering from polyneuritis. The skin defects usually developed two or three weeks after the outbreak of the disease and disappeared again after one or two months. In numerous patients showing this symptom Drs H. van Genderen of our Institute was unable to demonstrate any changes in the nicotinic acid content of the blood or urine.

From tables 5 and 6 it appears that, as usual, the cases of la-

TABLE 8.

Results of the toxin test with saliva from patients not suffering from diphtheria.

Patient no.	Initials of patient	Sex	Age in years	Nature of the disease	Results of the toxin test
51	P.S.	♂	22	Angina	negative
52	K.N.	♂	10	Angina	negative
53	H.P.H.	♂	11	Pseudo-croup	negative
54	F.W.	♂	2	Infectious mononucleosis	negative
55	G.H.E.	♀	29	Angina	negative
56	L.M.	♀	14	Angina	negative
57	v.d.N.M.	♀	41	Angina	negative
58	G.M.deH.	♀	25	Angina	negative
59	H.L.M.	♀	15.	Angina	negative
60	B.N.	♀	48	Angina	negative

ryngeal, nasal and cutaneous diphtheria did not show any complications.

Lastly, mention should be made of the fact that the same toxin test was carried out with saliva from ten patients in whose case the original diagnosis "diphtheria" was later proved as erroneous (the bacteriological test giving a negative result).

Some data concerning these cases are shown in table 8.

DISCUSSION.

Before entering upon a more detailed discussion of the obtained results we wish to point out that these toxin tests were only of a very inexact quantitative character. The terms "strong positive", "positive", "light positive" and "negative" only indicate very wide differences.

The expectation expressed in a previous publication (TASMAN and SMITH, 1954), i.e. that under certain favourable conditions it should be possible to demonstrate the presence of diphtheria toxin in the saliva of diphtheria patients, has been borne out by our practical results.

A careful study of the obtained results reveals that there is no correlation between the positive or negative results of the toxin test and the severity of the clinical symptoms or the course taken by the disease. In several mild cases the saliva of the patients in question (patients No. 6, 10, 13, 14, 16, 22, 23, 37, 40) gave a positive or strongly positive toxin reaction, whereas the saliva of several patients who showed a serious clinical picture or who died of the disease only gave a light positive or even a negative toxin reaction (patients No. 2, 18, 25, 27, 28, 31, 34, 36, 39, 41, and 45).

It is, however, obvious that there is a close connection between the result of the toxin test and the moment when the patient's saliva was collected. This is particularly evident if the results of the toxin test are related to the moment when the serum was injected. This correlation is clearly demonstrated in table 9.

Naturally, one should not attach too much value to the percentages of the positive results given here, in view of the small number of observations.

It is, however, clear that the chance of a positive toxin reaction

TABLE 9.

Correlation between the results of the toxin test and the injection of anti-diphtheria-serum

Saliva collected:	Number of cases	Results of the toxin test		% positive
		positive	negative	
Previous to the injection of serum	15	11	4	73
Immediately after the injection	8	5	3	63
One day after the injection	19	7	12	37
Two or more days after the injection	6	1	5	16

decreases as the time between inoculation and the collection of the saliva increases.

As already shown, there is no direct connection between the seriousness of the disease and the result of the toxin test; but one of the possible explanations of the above phenomenon still remaining was that it might be due to an excretion of diphtheria-antitoxin through the salivary glands.

This possibility was investigated. 20.000—50.000 A.U. of anti-diphtheria serum were injected intramuscularly into 5 patients, most of whom had been admitted into hospital as suspected of diphtheria, a diagnosis which shortly after their admission in most of these cases was found to have been wrong (among other things because the bacteriological test yielded a negative result). After some time we succeeded in demonstrating the presence of diphtheria antitoxin in the saliva of all 5 patients. In some of these cases the antitoxin tests of saliva and serum were checked on several occasions. Furthermore, the urine of 2 patients was found to contain diphtheria antitoxin (see Table 10). Table 11 gives the results obtained with the saliva and the serum of eight actively immunized healthy persons, in whose saliva no antitoxin was ever found.

This phenomenon will be dealt with in full detail in another publication. Here, however, we may already draw the conclusion that the intramuscularly injected heterologous diphtheria antitoxin (enzymatically purified and concentrated diphtheria plasma, obtained from horses) can be excreted after a certain time in small quantities through the salivary glands into the saliva and through the kidneys into the urine, in contrast with the homologous antitoxin, produced as a result of active immu-

TABLE 10.

Excretion of intramuscularly injected diphtheria-antitoxin in saliva and urine.

Patient no.	Initials of patient	Sex	Age in years	A.U. injected	Time elapsed after injection	Titre of blood serum (AU/ml)	Titre of saliva (AU/ml)	Titre of urine (AU/ml)
61	M.C.W.v.d.G.	♀	25	20.000	24 hours	—	<0.001	—
					48 ,,	3	0.002	—
62	M.W.K.	♀	15	20.000	14 ,,	7	negative	—
					38 ,,	7	0.003	—
					3 days	7	0.006	—
					4 ,,	7	0.006	—
					5 ,,	6½	0.020	—
63	W.H.P.	♀	20	20.000	17 hours	0.6	0.006	—
					2 days	2	0.008	—
					5 ,,	2	0.006	—
					12 ,,	1	0.006	—
					19 ,,	0.5	0.004	—
					45 ,,	<0.1	negative	—
64	W.J.K.	♂	14	40.000	4 hours	0.8	negative	doubtful
					24 ,,	3.5	0.003	0.04
					5 days	4.5	0.017	0.01
					11 ,,	4	<0.003	<0.003
					30 ,,	0.25	negative	negative
65	G.v.d.Z.	♂	5½	50.000	22 hours	10	0.034	0.055
					5 days	14	0.090	0.050

TABLE 11.

Examination of the saliva of actively immunized persons

No.	Initials of patient	Sex	Age in Years	Blood-serum titre (AU/ml)	Results obtained when testing saliva for antitoxin
1	S.L.	♀	4	0.01	negative
2	C.J.S.	♂	7	22	,,
3	A.P.v.d.B.	♂	8	18	,,
4	T.J.A.d.H.	♂	42	25	,,
5	M.d.H.	♀	32	2	,,
6	J.C.V.	♀	5	1.75	,,
7	A.R.	♀	7	8.5	,,
8	J.d.R.	♂	6	4	,,

nization, of which we could never find any trace in the saliva of the persons we examined.

We also wish to draw attention to the comparatively slow resorption of the intramuscularly injected diphtheria antitoxin, owing to which the maximal serumtitre sometimes is not reached until a considerable time after the injection (see especially patients Nos 63, 64 and 65). SNEATH (1934), GOLD and BACHERS (1943) and TURNER, STAFFORD and GOLDMAN (1954) observed a similar phenomenon in the case of intramuscularly injected tetanus antitoxin. This seems to lead to the very important practical conclusion, that it is indeed advisable, when injecting antitoxin intramuscularly into diphtheria patients, to make the doses very large. In all probability the rate of resorption, *i.e.*, the attainment of a sufficient serum titre, will be proportionate to the quantity of antitoxin injected. We even wonder whether it would not be better for every diphtheria patient to be given his first serum injection intravenously. In due time we hope to publish some facts bearing on this matter.

Although the quantities of diphtheria antitoxin found in the saliva of the patients in table 10 are very small in themselves, they must yet be considered amply sufficient to neutralize the quantities of diphtheria toxin which, but for the simultaneous presence of this antitoxin in the saliva, would cause a clearly positive reaction. Of course this excretion of antitoxin through the salivary glands (and the kidneys) requires some time to get going. Therefore it is but natural that the greatest number of positive toxin reactions will be found in the saliva collected from patients who had not yet had a serum injection. Shortly after this injection, too, the chance of the saliva showing a positive toxin reaction is still fairly great. This chance decreases, however, as the interval between the injection of the antitoxic diphtheria-serum and the collection of the saliva grows larger.

For the correct appreciation of the positive or negative result of the toxin test, one should moreover bear in mind that only part of the saliva produced by the patients has remained in the mouth for any length of time without being disturbed and that there will always be an admixture of saliva that is newly produced at the moment of collection, so-called "forced" saliva, which acts as a (in our case undesirable) diluent.

For the comparatively large quantities of diphtheria toxin which may be found under certain conditions, we may instance the case of patient No. 14. This girl was asleep when we wanted to collect her saliva. By the method described in the beginning of this paper, using a tampon, we took about 0.2 ml of saliva from her. This saliva, absorbed in saline, showed a "strong positive" toxin reaction. We found that at the moment of collection the saliva of this girl, removed from the oral cavity (cheek-pouch), contained about 0.1 guinea-pig M. L. D. per ml. This is a very considerable quantity! In this connection we may quote MUELLER (1941):

"From another point of view one is justified in assuming that no more than minimal quantities of toxin are produced in the actual disease. Those data which are available indicate that the susceptibility to diphtherial toxin is, weight for weight, about ten times that of a guinea-pig. The fatal dose for a child would therefore probably be less than ten guinea-pig M.L.D. . . . . "

In the beginning of this article we mentioned that we made every possible effort to prevent the patients from having any food or drink shortly before the collection of the saliva. Unfortunately, this did not always prove possible. This means, that in such cases some of the saliva that contained toxin was swallowed and replaced by quite new saliva, which contained no toxin and acted only as an undesirable diluent. We believe that we are justified in assuming that the percentage of positive toxin finds would have been considerably larger, especially in the case of those patients who had not yet had a serum injection when their saliva was collected, if the above-mentioned measure could have been more rigidly enforced. In this connection we may add that we obtained some "strong positive" toxin reactions with saliva from very "new" patients, in whose case the diagnosis "diphtheria" was only a provisional one and was not confirmed until one or two days later (among other things by a positive bacteriological test-culture).

For these reasons we believe that for saliva a very important place in the pathogenesis of diphtheria can be claimed.

This conclusion, and especially the fact, that heterologous diphtheria toxin, when administered parenterally, was found to be excreted fairly quickly through the salivary glands, though only in small quantities, underlines once more how important it is that patients suspected of diphtheria should be given a large dose of antitoxin diphtheria serum at the earliest opportunity. The minute quantities of antitoxin which we could demonstrate in the saliva of some patients who had been treated with serum suffice to neutralize relatively large quantities of toxin. We hope in due course to publish a report on another method of neutralizing the diphtheria toxin present in saliva.

The part played by saliva in the pathogenesis of diphtheria as a whole will also be discussed by us in another article.

Summary.

1. An inquiry into the possibility of demonstrating the presence of diphtheria toxin in the saliva of diphtheria patients was described.
2. With due observance of some precautions (the patients must abstain from food or drink for some time previous to the collection of the saliva) this proved to be indeed possible in a great number of cases.
3. The positive or negative result of the toxin test of the saliva was found to be independent of the clinical course of the disease.
4. Heterologous diphtheria antitoxin (enzymatically purified and concentrated diphtheria plasma obtained from horses), administered parenterally, was found to be excreted in small quantities after a certain time through salivary glands and kidneys into the saliva and the urine.
5. There appeared to be a close relation between the positive or negative result of the above-mentioned toxin reaction and the time elapsed between the moment when the serum was administered and that when the saliva sample was collected.
6. Under certain conditions relatively very large quantities of diphtheria toxin may be found. From a three-years-old girl, whom we found asleep, we took a saliva sample of only about 0.2 ml. This saliva proved to contain about 0.1 guinea-pig M.L.D. per ml.
7. In view of these results we stressed once more the importance of administering a large dose of antitoxic diphtheria serum at the earliest opportunity, if possible intravenously.

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UNE VIROSE A LOCALISATION INTESTINALE AVEC AFFINITE CYTOPLASMIQUE CHEZ LE LEPIDOPTERE *THAUMATOPOEA PROCESSIONEA*

par

C. VAGO et **L. VASILJEVIC**

(Reçu le 25 Septembre 1954).

Les viroses à corps d'inclusion polyédriques sont connues chez plus d'une centaine d'espèces de Lépidoptères. La pathogénèse de ces affections pathologiques est caractérisée par une affinité des virus Borrellina vis à vis des cellules sanguines, épithéliales et adipeuses. Les cellules péritrachéales sont souvent atteintes. Par contre les tissus musculaires et les tissus intestinaux ne sont pas attaqués (1, 2, 3).

Au cours de nos études sur la pathologie du lépidoptère *Thaumatopoea processionea* L. (processionnaire du chêne) nous avons remarqué dès 1952, a côté d'une virose à polyèdres normale (5) un autre type de polyédrie ayant des caractéristiques différentes de celles mentionnées ci-dessus.

Ces différences furent mises en évidence, d'une part, par examen microscopique de prélèvements de tissus, d'autre part, par histologie, et enfin par des essais de transmission artificielle.

METHODES.

Pour l'examen des prélèvements de tissus, ceux-ci furent mis sur frottis après ouverture de la cavité générale des larves. Les examens ont porté sur le tissu adipeux, le tissu trachéen et après dissection du tube digestif, sur les diverses sections intestinales. L'hémolymphe provient des ponctions opérées à la limite supérieure de la première fausse patte. Les altérations cellulaires ont été mises en évidence par colorations au May-Grunwald-Giemsa ou Wright ou encore par le bleu victoria qui accentue particulièrement les contours des corps d'inclusion (4). L'examen sur fond noir a été utilisé, mais le plus souvent nous avons eu recours au contraste de phase (6).

Les pièces histologique étaient fixées "in toto" ou l'intestin seul, dans du formol salé NaCl 6°/₀₀, dans du Bouin ou Bouin-Dioxane et furent inclus dans de la paraffine celloïdine ou Cremolan. Les colorations employées se complètent: hématoxyline ferrique — érythrosine pour obtenir un contraste des corps polyédriques, Kull pour les mitochondries, hémalun pour les structures nucléaires et bleu victoria dans le même but que pour les frottis.

HISTOPATHOLOGIE.

Notre attention fut attirée sur cette affection par le fait qu'à l'examen des prélèvements présentant les symptômes habituels (relachement musculaire, état flasque, mélanisation rapide) des polyédries, il est souvent impossible de trouver les corps d'inclusion non seulement dans l'hémolymphe mais également dans le tissu adipeux malgré l'emploi simultané de plusieurs techniques. Avec l'histologie il en est ainsi même pour les altérations chromatiniennes intra-nucléaires, caractérisant les cellules atteintes de polyédrie. Par contre, vers la lumière intestinale à partir de la membrane basale la majorité des cellules épithéliales mésointestinales sont hypertrophiées. Cette altération est souvent si prononcée que les cellules sont déformées et semblent fusionnées.

Ces cellules hypertrophiées contiennent un nombre variable d'éléments polyédriques pouvant aller jusqu'au remplissage total de la cellule. La forme de ces corps ressemble à celle des polyèdres de diamètre de 2 à 4 microns. Ils sont réfringents, réfractaires aux colorants d'aniline basiques. Leur contour se colore peu avec le bleu victoria et le violet de méthyle acidifié ou même au bleu de méthylène de Loeffler. Ils prennent l'acide picrique. Ces propriétés tinctoriales montrent une analogie très poussée avec celle des polyèdres classiques. Cependant il est à remarquer que l'hématoxyline ferrique ne pénètre qu'imparfaitement ces corps, alors qu'il colore en général les polyèdres dans les tissus adipeux.

Une autre différence importante vis à vis des polyédries habituelles est la localisation des corps d'inclusion. En effet, les polyèdres sont reconnus avoir une affinité intra-nucléaire: ils se forment à l'intérieur des noyaux, s'y accumulent et ne sont évacués dans le cytoplasme ou a l'extérieur des cellules que par rupture du noyau (1, 2, 3).

Or, dans la virose en question, les polyèdres se trouvent essentiellement dans le cytoplasme et souvent remplissent entièrement celuici. Les noyaux ne contiennent aucun élément semblable aux corps d'inclusion; ils restent hypertrophiés sans prendre les dimensions observées dans le cas des polyèdres intra-nucléaires.

A un stade avancé de la maladie, les corps d'inclusion se trouvent également dans la lumière intestinale bien que la rupture des cellules ne soit pas fréquente.

Virose experimentale.

L'ingestion expérimentale du broyat des cellules intestinales atteintes reproduit la maladie dans les individus sains dans les proportions de 42 à 68 %. La durée d'incubation semble plus courte que pour la polyédrie classique: à 22°C., au bout de 72 heures, les premiers signes de la formation des polyèdres apparaissent dans les cellules intestinales.

Cette polyédrie a conservé durant cinq passages contrôlés sa localisation intestinale sans présenter d'extension vis à vis des autres tissus, ce qui montre un net particularisme vis à vis des polyédries classiques.

Dans cet ordre d'idées il est intéressant de signaler que les cas où nous avons décélé la présence simultanée des polyèdres dans les tissus intestinaux et dans le tissu adipeux, le prélèvement des cellules intestinales atteintes n'a reproduit, "per os", que la forme localisée dans ce dernier type de cellules. D'autre part, nous avons obtenu par inoculation à partir du tissue adipeux des mêmes individus (bien qu'avec faible réussite) une reproduction de la polyédrie attaquant les cellules adipeuses et sanguines.

Ces faits semblent également signaler une certaine indépendance des deux types d'affection. L'étude de cette question se poursuit actuellement par purification et par comparaison des corps élémentaires au microscope électronique.

Conclusions.

Une virose à polyèdres a été mise en évidence sur *Thaumatopoea processionea* L. avec localisation dans le tissu intestinal alors que ce dernier est réfractaire aux polyédries en général.

D'autre part, contrairement au nucléotropisme des corps d'inclusion dans les polyédries habituelles, on constate ici, une affinité vis à vis du cytoplasme sans localisation nucléaire.

La transmission sélective fait penser à une certaine indépendance de la polyédrie des cellules intestinales par rapport à la polyédrie des cellules adipeuses et sanguines.

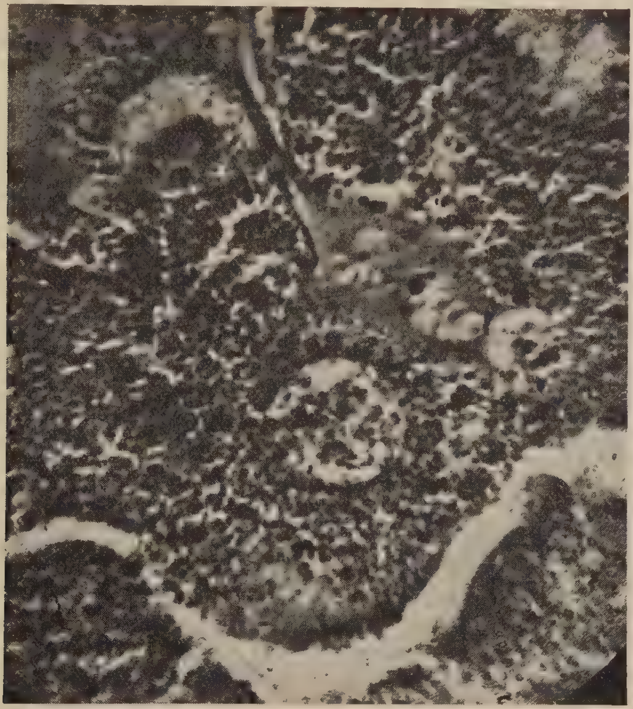

Fig. 1. Cellules intestinales virosées de *Thaumatopoea processionea* L. avec noyaux intacts et cytoplasme rempli de polyédres. Contraste de phase Wild. 1000 ×.

Zusammenfassung.

Eine von dem gewöhnlichen Type abweichende Polyädervirus-Krankheit wurde bei *Thaumatopoea processionea*-larven festgestellt.

Die Krankheit ist in den gegenüber den normalen Polyäderinfektionen widerstandsfähigen Darmzellen lokalisiert.

Die gewöhnlich nucleotropische Einschlusskörper sind hier ausschliesslich im Zellplasma angehäuft.

Eine selektive Ubertragung zeigt eine gewisse Unabhängigkeit der Intestinalpolyëderkrankheit von der Polyäderinfektion der Fett und Blutzellen. Die Fortsetzung der Arbeit bezieht sich auf elektronmikroskopische Untersuchung dieses Unterschiedes.

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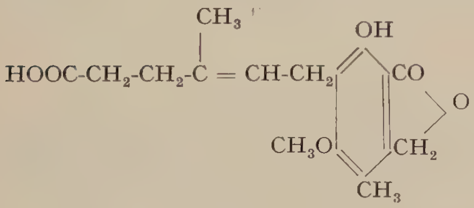
FILIATION BIOCHIMIQUE DES ACIDES PHÉNOLIQUES PRODUITS PAR *PENICILLIUM BREVI-COMPACTUM*

par

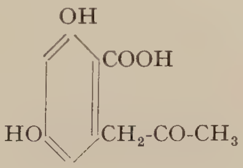
PAUL GODIN 1)

(Reçu le 1 Novembre 1954).

Depuis les travaux de l'école de RAISTRICK (2, 3, 6, 7, 12, 13, 14), on sait que les *Penicillium* du groupe *brevi-compactum* produisent, pour la plupart, cinq acides phénoliques identifiés par ces auteurs. Il s'agit de l'acide mycophénolique (I) : $C_{17}H_{20}O_6$, du 3,5-dihydroxy, 2-carboxybenzylméthylcétone (II) : $C_{10}H_{10}O_5$, du 3,5-dihydroxy, 2-carboxyphénylacétylcarbinol (III) : $C_{10}H_{10}O_6$, de l'hydrate de 3,5-dihydroxy, 2-carboxybenzoylméthylcétone (IV) : $C_{10}H_{10}O_7$ ou $C_{10}H_8O_6$–H_2O et de l'acide 3,5-dihydroxyphtalique (V) : $C_8H_6O_6$, tous corps dont les formules sont données ci-dessous.



(I)



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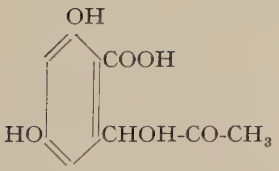

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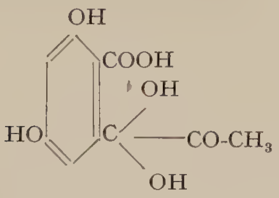
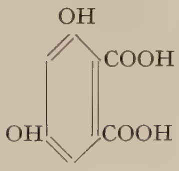


(IV)



(V)

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1) Chargé de Recherches du Fonds National de la Recherche Scientifique; Adresse actuelle: Laboratoire de la Sté Steinbach et Cie., Malmédy, Belgique.

Les trois substances en C_{10} renferment le même squelette carboné et diffèrent seulement par l'état d'oxydation de la chaîne latérale à trois atomes de carbone. La parenté chimique de ces trois corps entre eux et avec l'acide 3,5-dihydroxyphtalique est évidente. Mis à part l'acide mycophénolique dont la structure chimique est nettement différente, on peut supposer avec vraisemblance que, chez la moisissure, ces substances dérivent les unes des autres par suite d'oxydations ou de réductions éventuellement réversibles. Dans ces conditions, la synthèse de ces corps, au sens strict du mot, porterait d'une part sur l'acide mycophénolique et d'autre part sur un des composés en C_{10}.

Oxford et Raistrick (14) étudiant par des méthodes purement chimiques les relations biochimiques existant entre ces diverses substances phénoliques, sont arrivés aux conclusions suivantes: il ne semble pas exister de relation biochimique directe entre l'acide mycophénolique et les autres acides phénoliques; par contre il semble certain que $C_{10}H_{10}O_7$ provienne de l'oxydation de $C_{10}H_{10}O_6$ et que $C_8H_6O_6$ résulte de l'oxydation des acides phénoliques en C_{10}; quant à $C_{10}H_{10}O_5$, il pourrait provenir de la réduction de $C_{10}H_{10}O_6$.

Grâce aux méthodes chromatographiques et à la technique de substitution, on a pu vérifier et compléter ces résultats. On a notamment décelé dans le milieu de culture divers composés phénoliques nouveaux qui sont des produits de transformation des acides phénoliques connus.

Les méthodes expérimentales.

La moisissure étudiée est la souche Db182A de la mycothèque de Biourge du Laboratoire de Microbiologie de l'Université de Louvain, souche cataloguée comme *P. brevi-compactum*. Divers aspects du métabolisme de cette souche ont été décrits dans des travaux antérieurs (9, 10, 11).

Conditions de culture.

La moisissure est cultivée sur les milieux Czapek-Dox ou Raulin-Thom, à la température de 25°C. Après développement complet du microbe, on substitue au milieu de culture initial, la solution ou la suspension de la substance dont on veut étudier les transformations sous l'action de la moisissure. Les cultures se font en erlenmeyers de diverse capacité, maintenus immobiles (cultures stationnaires ou en surface) ou fixés au plateau d'un agitateur animé d'une rotation horizontale d'environ 100 circonvolutions par minute (cultures agitées ou immergées).

Obtention des acides phénoliques connus.

Les composés $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ et $C_8H_6O_6$ ont été extraits de la solution de métabolisme en appliquant les méthodes données par Clutterbuck et coll. (6). L'obtention de ces corps est décrite en détail dans une publication antérieure (10).

L'acide mycophénolique a été obtenu à partir du *P. brevi-compactum* de Biourge fourni par le Centraal Bureau voor Schimmelcultures de Baarn (Hollande). Cette souche, cultivée sur la solution de nitrate ammonique-glucose, milieu dont la composition est donnée par Fred et Waksman (8), ne produit pratiquement dans ces conditions que de l'acide mycophénolique. Après 6 semaines de culture, le pH du milieu est devenu alcalin et tout l'acide mycophénolique se trouve en solution dans le milieu. On filtre celui-ci, l'acidifie par H_2SO_4 et laisse déposer une nuit l'acide mycophénolique ainsi insolubilisé. On le recueille par filtration, le dissout dans l'alcool bouillant et le cristallise dans un mélange d'alcool et d'eau bouillante.

Le composé $C_{10}H_{10}O_5$ n'étant produit par la moisissure qu'en très minime quantité, a été préparé par réduction de $C_{10}H_{10}O_6$, suivant la méthode d'Oxford et Raistrick (13).

Analyse de la solution de métabolisme.

La solution de métabolisme est filtrée. On l'acidifie à pH inférieur à 3 par l'addition d'acide sulfurique dilué. On l'extrait ensuite à l'éther sulfurique, soit durant une nuit dans un extracteur continu, soit en 6 opérations successives, chaque fois avec son volume d'éther. La solution d'extraction est évaporée à sec. Le dépôt est dissous dans le minimum d'acétone et chromatographié. Si la solution d'extraction renferme de grandes quantités de $C_{10}H_{10}O_6$, on dissout le dépôt obtenu après évaporation de l'éther, dans le minimum d'acétone bouillant et déverse la solution acétonique dans 55 fois son volume de chloroforme bouillant. Par refroidissement, $C_{10}H_{10}O_6$ cristallise seul. On filtre la solution, l'évapore à sec sous pression réduite et reprend le dépôt par un peu d'acétone. Cette dernière solution est analysée par chromatographie. L'élimination de la solution à chromatographier, de la plus grande partie du $C_{10}H_{10}O_6$, élimination réalisée par le procédé de Clutterbuck et coll. (6), est motivée par le fait que cet acide représente à lui seul plus de la moitié de la quantité totale des composés phénoliques produits par la moisissure aux dépens du glucose. Si on ne se débarrasse pas de la plus grande partie de ce corps, sa concentration trop élevée dans la solution à analyser gêne la chromatographie.

La chromatographie des composés phénoliques est faite par la méthode ascendante, sur papier Whatman n° 4. Les solvants employés sont ou bien la couche surnageante d'un mélange de 5 volumes d'eau, de 4 volumes de n-butanol et de 1 volume d'acide acétique glacial (symbole : BAc), solvant proposé par BATE-SCHMITH (1), ou, de préférence, un mélange de 5 volumes de chloroforme et de 3 volumes d'acide acétique glacial, additionné de la quantité d'eau tout juste suffisante pour atteindre la saturation sans la dépasser (symbole : Chl). Les révélateurs sont : pour les acides, le vert de bromocrésol à 0,05% dans l'éthanol, pour les composés cétoniques, une solution saturée de 2,4-dinitrophénylhydrazine dans HCl 2 n (réactif de Brady), pour les substances phénoliques, une solution aqueuse de chlorure ferrique à 0,5%, le réactif de Pauly proposé par BOLLING et coll. (4) ou, de préférence, le réactif à la p. nitraniline de BRAY et coll. (5).

LES RÉSULTATS EXPÉRIMENTAUX.

Les substances phénoliques produites par la moisissure aux dépens du glucose.

On a renseigné dans des notes précédentes (9, 10) les acides phénoliques identifiés produits par la moisissure cultivée sur les milieux Czapek-Dox ou Raulin-Thom. Ce sont les cinq substances phénoliques trouvées par RAISTRICK et coll. Les caractéristiques chromatographiques de ces substances sont décrites dans des notes antérieures (9, 11).

En plus de ces corps dont la composition est connue, la moisissure produit en quantité minime six autres substances phénoliques non identifiées. On les désignera par les chiffres VI, VII, VIII, IX, X et XI, les chiffres I à V étant réservés aux acides phénoliques connus.

La substance VI donne une tache orangé-saumon avec le réactif de Pauly et celui à la p. nitraniline et une coloration pourpre amarante avec le chlorure ferrique. Elle est acide et cétonique. Rf dans BAc : 0,65, dans Chl : 0,13.

La substance VII donne une tache jaune intense avec le réactif de Pauly et celui à la p. nitraniline. Elle est faiblement acide, ne semble pas cétonique et ne réagit pas avec le chlorure ferrique. Rf dans BAc : 0,74, dans Chl : 0,22.

La substance VIII donne une tache jaune-saumon avec le réactif de Pauly et celui à la p. nitraniline et une teinte pourpre pâle avec le chlorure ferrique. Ce corps est acide et ne semble pas cétonique. Rf dans Chl : 0,75.

La substance IX donne une tache jaune pâle avec le réactif de Pauly, jaune canari avec celui à la p. nitraniline et une teinte pourpre pâle avec le chlorure ferrique. Elle n'est ni cétonique, ni acide au vert de bromocrésol. Rf dans Chl : 0,95.

La substance X ne réagit pas avec le réactif de Pauly ni avec celui à la p. nitraniline. Elle est cétonique et donne une tache pourpre rosé avec le chlorure ferrique. Elle n'est pas acide au vert de bromocrésol. Rf dans BAc : 0,17, dans Chl : 0,37.

La substance XI ne réagit ni avec le réactif de Pauly, ni avec celui à la p. nitraniline. Elle ne semble pas cétonique et n'est pas acide au vert de bromocrésol, mais donne une tache pourpre violet pâle avec le chlorure ferrique. Rf dans BAc : 0,415, dans Chl : 0,80.

Les substances X et XI ne sont décelées que dans l'extrait éthéré du milieu Raulin-Thom, milieu qui est d'ailleurs notoirement plus riche en acide mycophénolique que le milieu Czapek-Dox.

Tous ces composés, sauf l'acide mycophénolique et les substances X et XI, se trouvent également dans la solution de métabolisme après substitution au milieu de culture initial d'une solution de glucose à 5%.

Les substances phénoliques produites par la moisissure aux dépens de l'acide mycophénolique.

La moisissure est ensemencée sur 75 ml de milieu Czapek-Dox, dans des erlenmeyers de 150 ml de capacité dans le cas des cultures stationnaires, et de 500 ml de capacité dans le cas des cultures agitées. Après développement de la moisissure, on substitue au milieu de culture initial une suspension à 0,1% d'acide mycophénolique : 25 ml par culture en surface et 50 ml par culture agitée. En quelques jours d'incubation à 25°C., le dépôt d'acide mycophénolique est solubilisé par suite de la hausse du pH, plus rapide chez les cultures agitées (après 10 jours, pH 8,5) que chez les cultures en surface (après 10 jours, pH 7,6).

Dans la solution de métabolisme des cultures en surface, on ne décèle par chromatographie que l'acide mycophénolique. Par contre, dans le cas des cultures agitées, on met en évidence, outre l'acide mycophénolique, de faibles quantités des substances phénoliques X et XI. On sait par ailleurs que la moisissure cultivée sur le milieu Czapek-Dox produit des quantités d'acide mycophénolique plus grandes si la culture est agitée que si elle est immobile (10).

Les substances phénoliques produites par la moisissure aux dépens de l'acide phénolique $C_{10}H_{10}O_6$.

La moisissure est ensemencée dans des erlenmeyers de 500 ml de capacité, sur 200 ml de milieu Czapek-Dox dans le cas des cultures en surface et sur 75 ml du même milieu dans le cas des cultures agitées. Après développement de la moisissure, on substitue au milieu des cultures stationnaires, 10 ml d'une solution à 0,5% de $C_{10}H_{10}O_6$ et au milieu des cultures agitées, 50 ml de la même solution.

L'analyse chromatographique de la solution de métabolisme, tout autant des cultures en surface que des cultures agitées, révèle la présence de quantités abondantes de $C_{10}H_{10}O_6$ non métabolisé, de quantités moins abondantes de $C_{10}H_{10}O_7$, de quantités moindres encore de $C_8H_6O_6$ et des substances phénoliques VI et VII et enfin les substances phénoliques VIII, IX et $C_{10}H_{10}O_5$, toutes trois à concentration plus faible que les précédentes.

Les transformations subies par $C_{10}H_{10}O_6$ s'effectuent rapidement: en moins de deux jours dans le cas des cultures agitées et en moins de quatre jours dans le cas des cultures stationnaires. Passé ce délai, la concentration des différentes substances phénoliques produites ne se modifie plus sensiblement.

Les substances phénoliques produites par la moisissure aux dépens de $C_{10}H_{10}O_7$.

La moisissure est ensemencée sur 200 ml de milieu Czapek-Dox. On y substitue, après développement de la moisissure, 100 ml d'une solution à 0,5% de $C_{10}H_{10}O_7$.

Par chromatographie, on trouve dans la solution de métabolisme, en plus de notables quantités de $C_{10}H_{10}O_7$ non transformé, des quantités assez minimes de $C_{10}H_{10}O_6$ et de la substance VII, des quantités moindres de $C_8H_6O_6$ et de la substances VI et enfin, de très faibles quantités de $C_{10}H_{10}O_5$.

Les substances phénoliques produites par la moisissure aux dépens de $C_8H_6O_6$.

La moisissure est ensemencée sur 40 ml de milieu Czapek-Dox contenus dans un erlenmeyer de 100 ml de capacité. Après développement du mycélium, on substitue au milieu de culture initial, 15 ml d'une solution à 0,1% de $C_8H_6O_6$.

Après 6 et 12 jours d'incubation, on décèle dans la solution de métabolisme le composé $C_8H_6O_6$ en quantité notable et des traces de $C_{10}H_{10}O_6$ et de la substance VII. Ces dernières substances étaient présentes dans le mycélium dès avant la substitution et c'est vraisemblablement après cette substitution qu'elles ont diffusé dans la solution de métabolisme.

DISCUSSION.

L'acide mycophénolique et ses dérivés.

La première constatation qui s'impose, c'est que le métabolisme de l'acide mycophénolique diffère de celui des autres composés phénoliques en C_{10}, ce que OXFORD et RAISTRICK (14) avait déjà établi. La différence de structure chimique laissait d'ailleurs présager la chose. On remarque aussi que les substances phénoliques X et XI sont des produits de transformation de l'acide mycophénolique et n'interviennent pas dans la série de transformations dont les autres substances phénoliques sont l'objet. On vient en effet de voir que les composés X et XI ne sont jamais décelés lorsqu'on ne met pas l'acide mycophénolique en évidence, notamment dans les solutions de glucose substituées au milieu de culture initial complet. Qui plus est, ces composés sont produits par la moisissure aux dépens d'acide mycophénolique, alors qu'aucune des substances en C_{10} n'apparait dans la solution de métabolisme de ce même acide. Inversément, ni l'acide mycophénolique, ni les substances X et XI ne sont rencontrés dans les produits de transformation des acides phénoliques en C_{10} ou en C_8 expérimentés.

D'autre part, le fait que les substances X et XI ne réagissent pas avec le réactif de Pauly quoiqu'elles soient phénoliques (réaction avec le chlorure ferrique), prouve que les positions ortho et para par rapport au(x) groupement(s) phénolique(s) de leur molécule ne sont pas libres. Or, des substances phénoliques identifiées produites par la moisissure, seul l'acide mycophénolique présente cette caractéristique. On peut donc déduire de ceci qu'il existe vraisemblablement une parenté chimique étroite entre l'acide mycophénolique et les substances X et XI, parenté chimique due aux relations biochimiques existant entre ces corps.

Quant au processus de synthèse de ces trois corps, il est plus complexe que celui des autres substances phénoliques. Ces premiers corps ne sont notamment produits que si la moisissure incube au contact d'un milieu de culture complet, renfermant tous les éléments nécessaires au développement du microbe, tandis que les autres substances phénoliques sont également produites en abondance après substitution au milieu de culture initial d'une simple solution de glucose. Est-ce à dire que la synthèse de l'acide mycophénolique et des substances X et XI est sous la dépendance directe du métabolisme azoté de la moisissure alors que le processus de synthèse des autres composés phénoliques ne relève directement que du seul métabolisme glucidique? Il n'est pas encore possible de répondre à cette question.

Les autres substances phénoliques.

On vient de voir que le métabolisme de l'acide mycophénolique et de ses dérivés diffère de celui des substances phénoliques en C_{10} et de leurs dérivés. On a notamment démontré dans une publication antérieure (10) que le processus de synthèse de ces dernières substances fait partie du métabolisme glucidique général de la moisissure, sans directement dépendre de son métabolisme azoté.

Les résultats des recherches décrites ici permettent en plus d'affirmer que ces différentes substances dérivent les unes des autres par une série de transformations successives. En effet, en l'absence de toute autre source de carbone, la moisissure produit aux dépens du seul $C_{10}H_{10}O_6$, les substances phénoliques non identifiées VI, VII, VIII et IX et les composés $C_{10}H_{10}O_5$, $C_{10}H_{10}O_7$ et $C_8H_6O_6$. De même, à partir du seul $C_{10}H_{10}O_7$, la moisissure forme les composés $C_{19}H_{10}O_5$, $C_{10}H_{10}O_6$ et $C_8H_6O_6$ à côté des substances VI et VII.

En tenant compte du Rf des substances phénoliques non identifiées, notamment dans le solvant Chl, et en remarquant que les substances VI et VII sont produites par la moisissure aux dépens de $C_{10}H_{10}O_6$ et de $C_{10}H_{10}O_7$ alors que les substances VIII et IX ne sont obtenues qu'à partir du seul $C_{10}H_{10}O_6$ et cela, en très faible quantité, on peut supposer avec grande vraisemblance, d'une part, que les composés VI et VII sont des produits de l'oxydation de $C_{10}H_{10}O_7$ intermédiaires entre ce corps et $C_8H_6O_6$ et, d'autre part, que les substances VIII et IX sont des produits de réduction de $C_{10}H_{10}O_6$, moins oxydés encore que $C_{10}H_{10}O_5$.

Il est donc très vraisemblable que ces différentes substances constituent des intermédiaires du processus d'oxydation figuré ci-après, amenant par étapes successives la substance IX, la moins oxydée, à l'état de $C_8H_6O_6$, le corps final de la série: IX → VIII → $C_{10}H_{10}O_5 \rightarrow C_{10}H_{10}O_6 \rightarrow C_{10}H_{10}O_7 \rightarrow$ VII → VI → $C_8H_6O_6$.

Certaines étapes de ce processus sont certainement réversibles et il n'est pas encore possible de dire quelle est la substance de cette série qui est produite en premier lieu par la moisissure, c'est à dire quelle est celle dont toutes les autres dérivent, soit par réduction, soit par oxydation.

Résumé.

Le *P. brevi-compactum* produit onze substances phénoliques différentes: l'acide mycophénolique, les acides phénoliques $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ et $C_8H_6O_6$ et les substances phénoliques non identifiées désignées par les chiffres VI, VII, VIII, IX, X et XI. L'acide mycophénolique et les substances X et XI qui en dérivent, sont synthétisés par la moisissure suivant un processus plus complexe que les autres substances phénoliques et sans rapport direct avec lui. Ces dernières dérivent les unes des autres par une série de transformations dont certaines sont réversibles. Il semble que les substances VI et VII soient des intermédiaires entre $C_{10}H_{10}O_7$ et $C_8H_6O_6$, tandis que les substances VIII et IX seraient des produits de réduction de $C_{10}H_{10}O_5$.

Summary.

P. brevi-compactum produces eleven phenolic different substances, mycophenolic acid, phenolic acids $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$, and the non-identified substances designed by the numbers VI, VII, VIII, IX, X and XI. Mycophenolic acid and its derivates X and XI are synthesized by the mould according to a process more complex than the other phenolic substances and have no direct connection with them. The latter derive the one from the other, in a succession of transformations some of which are reversible. It seems that the substances VI and VII are intermediaries between $C_{10}H_{10}O_7$ and $C_8H_6O_6$, the substances VIII and IX being produced by reduction of $C_{10}H_{10}O_5$.

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OXIDATION OF TRICARBOXYLIC ACID CYCLE INTERMEDIATES BY *STREPTOMYCES NITRIFICANS* ¹)

by

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INTRODUCTION.

In studies on the intermediary metabolism of heterotrophic microflora, far more work appears to have been done with bacteria, yeasts, and molds than with actinomycetes (WAKSMAN, 1947, 1950). Although the occurrence, morphology, and cultural characteristics of these organisms have been studied in some detail (LIESKE, 1921), their industrial value has only recently stimulated investigation of their intermediary metabolism (PERLMAN, 1953). With respect to the tricarboxylic acid cycle, actinomycetes are not mentioned in the reviews of AJL (1951) or KREBS (1954). However, COCHRANE and PECK (1953) considered this pathway to function in *Streptomyces coelicolor*. GARNER and KOFFLER (1951) questioned whether *Streptomyces griseus* operated via a Krebs cycle, but HOCKENHULL *et al.* (1954) as well as COTY (1954) observed that this organism metabolized tricarboxylic acid cycle intermediates. Various mycobacteria apparently possess a citric acid terminal respiratory cycle (MILLMAN and YOUMANS, 1954), despite the inability of intact cells of some species to oxidize Krebs cycle intermediates (EDSON, 1951; HOLMGREN *et. al.*, 1954).

Because the wide range of enzymes blocked by urethan (ethyl carbamate) includes succinic dehydrogenase (SVENSSON, 1923; SEN,

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1931; Thunberg, 1936), one may expect this substance to interfere with the tricarboxylic acid cycle. According to Yosue and Iida (1951), streptomycin-resistant tubercle bacilli lacked this enzyme. Dinning *et al.* (1950) found that succinic dehydrogenase activity was subnormal in the bone marrow of mature hens treated with ethyl carbamate. Bernelli-Zazzera (1951) also noted an inhibitive effect of urethan on the succinic dehydrogenase of rat origin. However, Shimakoshi (1953) considered that the effects of urethan on various rat brain dehydrogenases was due to interference with the redox dyes employed in the Thunberg technic rather than to direct action on the enzymes themselves.

This paper reports studies on the oxidation of tricarboxylic acid cycle intermediates by *S. nitrificans*. These investigations were carried out in an effort to obtain information on the mode of action of urethan. This actinomycete may provide a useful approach to this problem since it is uniquely capable of growing on urethan as the sole source of nitrogen, carbon, and energy (Schatz *et al.*, 1954). Any difference or induced derangement in the area of tricarboxylic acid cycle metabolism might conceivably shed light on the mechanism of urethan action. An understanding of the biochemistry of this compound is desirable because of its important physiological properties; e.g. urethan functions as a mutagen, carcinogen, leukopenic agent, narcotic drug, and enzyme inhibitor. Despite extensive physiological and pharmacological research, relatively little is yet known of the precise mode(s) of action responsible for the extraordinary behavior of so simple a compound.

MATERIALS AND METHODS.

S. nitrificans was grown in the following synthetic mineral solution as a basal medium:

KH_2PO_4	0.05%
$MgSO_4 . 7H_2O$	0.02%
Fe (as $FeCl_3 . 6H_2O$)	0.4 mg%
Ca (as $CaCO_3$ plus minimal HCl to dissolve)	2.5 mg%
Mn (as $MnCl_2 . 4H_2O$)	0.2 mg%
Zn (as $ZnSO_4 . 7H_2O$)	0.04 mg%
Mo (as $Na_2MoO_4 . 2H_2O$)	0.02 mg %
Cu (as $CuSO_4 . 5H_2O$)	0.002 mg%
Co (as $CoCl_2 . 6H_2O$)	0.02 mg%
Ethylenediaminetetraacetate	0.02%

pH adjusted to 6.8 to 7.0 with NaOH.

Ethylenediaminetetracetic acid served as a non-toxic, non-metabolizable chelating agent for maintaining trace metals in a readily available form (Hutner *et al.*, 1950). This was especially advantageous in the urethan medium which became markedly alkaline due to NH_3 liberated during growth. Because of the rise in pH, precipitation of essential trace metals as insoluble phosphates occurred in the absence of a sequestering agent. To grow U-cells [1]), the basal medium was supplemented with 0.2% urethan as a source of nitrogen, carbon, and energy. Since growth of *S. nitrificans* on this carbamate was very slow, the one liter Erlenmeyer flasks, each containing 150.0 ml, were incubated 23 days at 28—30°C. PGY-cell [2]) material was harvested from 4 day old shake cultures of 0.5% peptone, 0.5% glycerol, and 0.2% yeast extract in the basal synthetic solution dispensed 100.0 ml per 250 ml Erlenmeyer flask.

To recover cell material after incubation, the cultures were filtered by suction onto paper in a Buchner funnel and washed several times with distilled water. The damp mycelium thus recovered was first suspended in distilled H_2O and homogenized by a rapidly rotating Teflon pestle in a Pyrex mortar at 0°C. The resulting thick homogenate was then diluted with M/15 Sörensen's phosphate buffer at pH 7.0, and a double strength basal mineral solution prepared phosphate-free for this purpose. Each cell homogenate was finally made up in 0.024 M phosphate at pH 7.0 and contained 72.7% of the minerals that were present in the synthetic basal medium in which the organism had grown (Mohan *et al.*, 1955). Respiration substrates were prepared in similar solutions to avoid changes in pH, buffer, and trace metal concentration upon tipping. The Warburg vessels routinely contained KOH in the center well and 1.8 or 1.4 mg of cell nitrogen for PGY- or U-cells, respectively. In experiments with PGY-cells, substrates were tipped after 60—90 minutes of shaking in the 30°C. water bath. This preaeration decreased the endogenous respiration of PGY homogenates by approximately 50%. U-cells exhibited much less autorespiration and were therefore not preaerated.

EXPERIMENTAL RESULTS AND DISCUSSION.

The results presented in figures 1 and 2 show that *S. nitrificans* readily respired the 2-, 4-, and 6-carbon intermediates of the tricarboxylic acid cycle. From the data in table 1 calculated for these curves prior to their breaks, it is apparent that U-grown cells consumed O_2 at decreasing rates for acetate, citrate, succinate, fumarate, malate, *trans*-aconitate, and *iso*-citrate in the order given. For PGY-grown material, the corresponding arrangement of substrates was: succinate, fumarate, acetate, citrate, malate, *trans*-aconitate, and *iso*-citrate. Unfortunately, it was not learned until after these studies were completed that the aconitic acid used was the *trans* rather than the *cis*-isomer. However, the fact that the

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[1]) „U" refers to growth on urethan.

[2]) „PGY" represents *S. nitrificans* cultured on peptone, glycerol, and yeast extract.

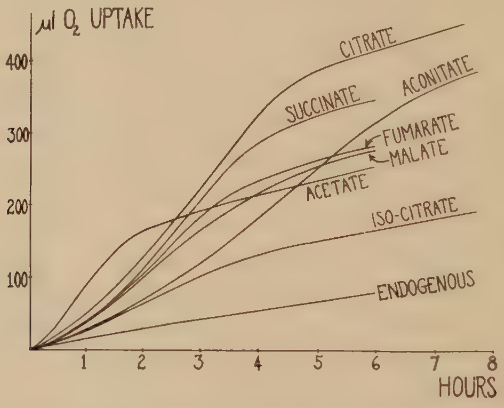

Fig. 1. Oxidation of 2-, 4-, and 6-carbon compounds by *S. nitrificans* grown on urethan as the sole source of carbon, nitrogen, and energy.

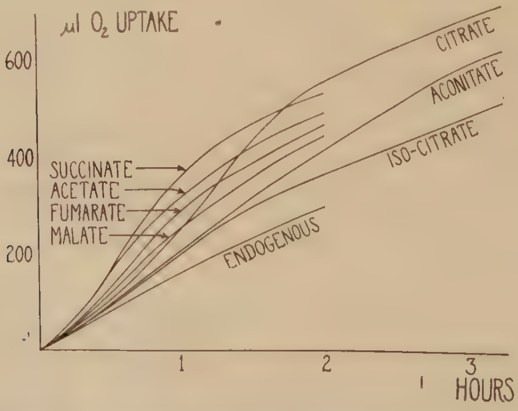

Fig. 2. Oxidation of 2-, 4-, and 6-carbon compounds by *S. nitrificans* grown on peptone, glycerol, and yeast extract.

trans form was oxidized at an appreciable rate suggests that the biologically active *cis*-aconitate would probably be even more rapidly respired.

The calculations in both tables 1 and 2 have been made on the assumption that endogenous respiration continued unchanged during substrate oxidation. This was actually shown to occur with 10.0, 20.0, 30.0, and 40.0 μM of succinate which were all oxidized at the same rate. Moreover the curves broke successively at equal intervals so that the percent of oxidative assimilation was identical for the four concentrations of succinate when calculated on the basis of steady and unaltered autorespiration. Although not shown in figures 1 and 2, the O_2 uptake was increased only very slightly (3 to 5%) over the endogenous level when 10.0 μM of pyruvate or α-ketoglutarate were supplied as respiration substrates. At this same concentration, glutamate was rapidly oxidized, but α-alanine alone or together with α-ketoglutarate was metabolized very slowly. The influence of α-alanine was tested because of possibly converting α-ketoglutarate to glutamate via transamination, a reaction known to occur in a number of bacteria (LICHSTEIN and COHEN, 1944). With respect to these compounds, however, *S. nitrificans* behaved similarly whether grown on urethan or the PGY medium. Since fairly large amounts of cell material were present in each Warburg vessel, it is unlikely that a deficiency of essential co-factors limited oxidation or amination of the α-keto acids. This belief was supported by the failure of co-carboxylase, methylene blue, and 2,6-dichlorophenol indophenol, when added as electron acceptors, to increase appreciably the gas uptake. Respiration of 10.0 μM of α-ketoglutarate was also not stimulated by 0.1 μM of glutamine, a compound necessary for the utilization of α-ketoglutarate by *Lactobacillus arabinosus* (AYENGAR *et al.*, 1951). It is not yet known whether pyruvate and α-ketoglutarate are decarboxylated by *S. nitrificans* under these conditions where oxidation occurs slightly if at all. When grown for 10 days in stationary culture on 0.2% Na_3 citrate . $2H_2O$ and 0.05% $(NH_4)_2SO_4$, *S. nitrificans* oxidized α-ketoglutarate somewhat more readily. After correction for autorespiration, the O_2 uptake for 10.0 μM of substrate was: succinate 217 μl, fumarate 179 μl, malate 154 μl, acetate 110 μl, α-ketoglutarate 41 μl, and pyruvate 19 μl. In the same 230 minute interval, 37 μl of O_2 were consumed endogenously.

The observations recorded in figure 3 suggest that pyruvate

TABLE 1.

Respiration of tricarboxylic acid cycle compounds by *Streptomyces nitrificans.*

Substrate 10 μM	Cells grown on	Calculations for entire curves 1)				Calculations up to breaks in curves 1)			
		μl O_2 uptake per minutes	Q_{O_2} (N) 2)	% decrease in Q_{O_2} (N) 3)	% substrate oxidatively assimilated	μl O_2 uptake per minutes	Q_{O_2} (N) 2)	% decrease in Q_{O_2} (N) 3)	% substrate oxidatively assimilated
Acetate	PGY	186/120	52	58	58	158/70	75	27	65
	U	177/360	22		60	139/110	55		69
Succinate	PGY	234/120	65	6	70	222/70	105	59	72
	U	265/360	61		66	229/230	43		71
Fumarate	PGY	164/120	45	46	76	138/60	77	59	80
	U	204/360	24		70	159/215	31		77
Malate	PGY	141/120	39	38	79	101/60	56	46	85
	U	203/360	24		70	152/215	30		77
Citrate	PGY	288/195	50	30	71	246/110	75	36	76
	U	379/465	35		62	299/270	48		70
Iso-citrate	PGY	83/195	14	20	92	62/80	26	46	94
	U	114/165	11		89	82/255	14		92
Trans-aconitate	PGY	199/195	34	17	80	176/165	35	20	82
	U	302/465	28		70	234/360	28		77
Autorespiration (control)	PGY	412/195	71	86					
	U	108/465	10						

1) Calculations for curves in figures 1 and 2. Values shown have been corrected by subtracting autorespiration data.

2) Q_{O_2} (N) = μl O_2 consumed per hour by mycelium homogenate containing 1.0 mg of cell nitrogen.

3) Percent decrease = $\frac{Q_{O_2}\text{ (N) for PGY minus }Q_{O_2}\text{(N) for U}}{Q_{O_2}\text{ (N) for PGY}} \times 100.$

TABLE 2.

Oxidation of α-keto acids by *Streptomyces nitrificans*.

Substrate	Cells grown on	Calculations for entire curves[1]			Calculations up to breaks in curves[1]		
		μl O_2 uptake per minutes	Q_{O_2} (N)[2]	% substrate oxidatively assimilated	μl O_2 uptake per minutes	Q_{O_2} (N)[2]	% substrate oxidatively assimilated
Pyruvate (60 μM)	PGY	185/210	30	94	181/150	40	95
Pyruvate (90 μM)	U	596/200	127	88	570/140[3]	171[3]	89[3]
					359/50 [3]	307[3]	93[3]
α-Ketoglutarate	PGY	69/210	11	99	67/75	29	99
(120 μM)	U	138/200	29	98	87/50	75	99
Autorespiration	PGY	220/210	35				
(control)	U	64/200	13				

[1] Calculations for curves in figures 4 and 5. Values shown have been corrected by subtracting autorespiration data.

[2] Q_{O_2} (N) = μl O_2 consumed per hour by mycelium homogenate containing 1.0 mg of cell nitrogen.

[3] These duplicate data are for the two points A (Q_{O_2} = 171) and B (Q_{O_2} = 307) at which this curve breaks (figure 4).

utilization may be sparked by the simultaneous oxidation of other substrates. After correcting for autorespiration, the O_2 uptake for pyruvate (12.0 μM), glycerol (8.0 μM), and succinate (8.0 μM) when supplied individually to PGY-cells was 25, 120, and 195 μl, respectively. With no sparking action, the utilization of pyruvate and glycerol together would be expected to consume $25 + 120 = 145$ μl O_2, whereas actually 195 μl O_2 were taken up which is 32% more than the calculated value. In the case of pyruvate plus succinate, the observed 306 μl O_2 uptake is 39% above the additive value of $25 + 195 = 220$ for these two compounds when they are independently metabolized. If the increased O_2 uptake is due to respiration of pyruvate, then the oxidative assimilation for pyruvate alone, for pyruvate in the presence of glycerol, and for pyruvate in the presence of succinate would be 96, 93, and 89%, respectively. These values correspond well with those reported in table 2 for much higher concentrations of pyruvate. Nevertheless, such findings taken by themselves do not conclusively establish sparking. One other possible explanation is that pyruvate, without being itself utilized, may in some way decrease the degree of oxidative assimilation of glycerol and succinate, and in this way be responsible for the appreciably greater O_2 uptake that is observed. It is unlikely that pyruvate functions, in addition to O_2, as a hydrogen acceptor to form lactate during respiration of glycerol and succinate. If this occurred, then a lesser gas consumption would be associated with the presence of pyruvate whereas just the reverse is observed. Moreover, higher concentrations of pyruvate are rapidly respired, as

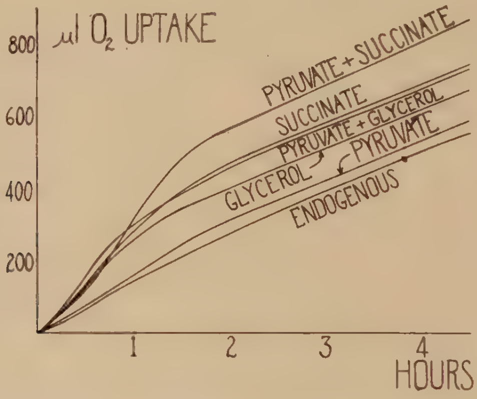

Fig. 3. Suggested sparking of pyruvate utilization by glycerol and succinate.

indicated in table 2, and at rates greater than for lactate (MOHAN *et al.*, 1955).

If pyruvate utilization were indeed sparked by glycerol and succinate, this would tend to rule out pyruvate as the end-product of succinate oxidation. The possibility that *S. nitrificans* might convert succinate to oxalacetate and then via decarboxylation to pyruvate had originally been considered. This was suggested by the decreasing rates of oxidation of succinate, fumarate, and malate at 10.0 μM levels and also by the relative inertness of pyruvate at the same concentration. The formation of various acids has been observed by other actinomycetes (PERLMAN, 1953). *Streptomyces griseus* in particular produces α-keto acids, among which pyruvate is the most abundant, from succinate and other substrates including certain tricarboxylic acid cycle intermediates (HOCKENHULL *et al.*, 1954). *Mycobacterium butyricum* also produces α-keto acids, especially α-ketoglutarate (HUNTER, 1953). In the case of *S. nitrificans*, the 222 and 229 μl O_2 consumed during succinate oxidation by cells grown on PGY and U, respectively, are close to the 224 μl required for quantitative conversion to pyruvate. But the fumarate and malate data differ appreciably from the 112 μl O_2 theoretical value for each of these substrates.

The low gas uptake with 10.0 μM of pyruvate and α-ketoglutarate was not attributed to penetration difficulty (UMBREIT, 1953) since the larger molecular-sized 6-carbon compounds were oxidized more rapidly when supplied at the same molar concentration. Moreover, the cell homogenates had been frozen overnight prior to use, and since this procedure ruptures cell membranes (HUGO, 1954) it was considered unlikely that the α-keto acids failed to penetrate due to permeability obstruction. Despite this, concentrations much greater than 10.0 μM were tried. The results (figures 4 and 5) prove that at higher levels these substrates were readily respired. Both U- and PGY-grown cells oxidized pyruvate more rapidly than α-ketoglutarate. Even at 120.0 μM, the latter was not respired as readily as 30.0 μM of pyruvate.

Streptomyces coelicolor (COCHRANE and PECK, 1953) and *Streptomyces griseus* (HOCKENHULL *et al.*, 1954) have also been shown to oxidize pyruvate more rapidly than α-ketoglutarate. But those results are not directly comparable with the present findings because of differences in substrate concentration as well as other conditions. The pyruvate curves for 60.0 and 90.0 μM are especially interesting since each of these exhibited two breaks, a phenomenon not observed with α-ketoglutarate even at the 120 μM level. This indicates that pyruvate is probably first oxidized to a product which temporarily accumulates but is subsequently respired at a slower rate. From the 90.0 μM pyruvate curve it is calculated that the direct or immediate oxidation of this substrate itself consumed 359 μl O_2 whereas respiration of the intermediate product resulted in an additional O_2 uptake of 211 μl. The overall oxidation of 90.0 μM of pyruvate therefore consumed 570 μl O_2. A 2-step utilization was also observed for pyruvate at 60.0 but not at 30.0 μM.

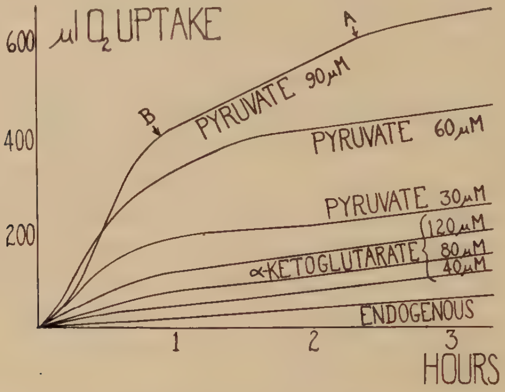

Fig. 4. Oxidation of α-keto acids by *S. nitrificans* grown on urethan.

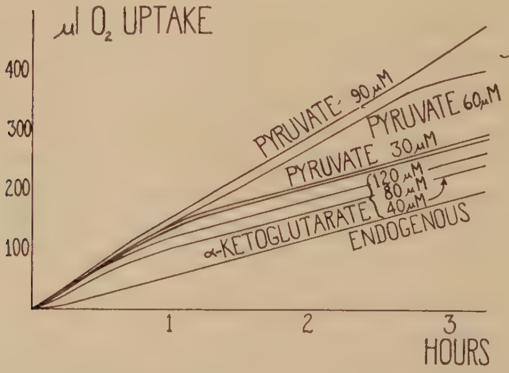

Fig. 5. Oxidation of α-keto acids by *S. nitrificans* grown on peptone, glycerol, and yeast extract.

Calculations of the data obtained from respiration studies show a marked difference in the ability of *S. nitrificans* to oxidize the 2-, 4-, and 6-carbon compounds of the tricarboxylic acid cycle as compared with the 3- and 5-carbon α-keto acids. The results presented in table 1 reveal that with acetate, succinate, fumarate, malate, citrate, *iso*-citrate and *trans*-aconitate the $Q_{O_2}(N)$ values were significantly higher for PGY-cells than for U-grown material. But according to the data in table 2, the reverse held true with pyruvate and α-ketoglutarate. Here the $Q_{O_2}(N)$ values were much greater for U- than for PGY-cell material. This variation in respiration rates, on a percentage basis, was entirely unrelated to the $Q_{O_2}(N)$ data for either the PGY or U medium. It is interesting that in spite of this difference, for which no explanation can yet be offered, the percentage of each substrate oxidatively assimilated is of the same magnitude for *S. nitrificans* grown on urethan as compared with the PGY-medium. One might assume that U-cell material would oxidatively assimilate more substrate because of its low autorespiration indicating a lack of reserve food or a normally starved condition (MOHAN *et al.*, 1955). This supposition rested on the far greater autorespiration characteristic of PGY-mycelium. The high endogenous oxidation of this actinomycete harvested from PGY medium approximates the high rates reported for *Streptomyces lavendulae* (WOODRUFF and FOSTER, 1943), *Streptomyces coelicolor* (COCHRANE and GIBBS, 1951), and *Streptomyces griseus* (HOCKENHULL *et al.*, 1954). The low autorespiration of U-grown *S. nitrificans* therefore appears unusual for this genus. The thermophilic *Micromonospora vulgaris* exhibited a low endogenous respiration but this was observed at 60°C. (ERIKSON and WEBLEY, 1953). The difference between the autorespiration of PGY- and U-material is actually twice as great as indicated in tables 1 and 2 because of the preaeration of PGY- but not U-cells. The greater endogenous $Q_{O_2}(N)$ in table 1 as compared with the corresponding value in table 2 probably results from variation in preaeration to effect starving.

Because of the already-cited reports on the inhibition of succinic dehydrogenase by urethan, studies were carried out to determine whether this occurred in the case of *S. nitrificans*. The results, which will be reported in detail elsewhere (SCHATZ *et al.*, to be published), showed that 30.0 μM of urethan did not affect appreciably either the rate or the percent of oxidative assimilation of 10.0 μM of succinate by PGY-cell material, which did not respire the carbamate.

Likewise, for U-grown *S. nitrificans*, the final O_2 uptake with both urethan (20.0 μM) and succinate (10.0 μM) supplied together was equal to the total gas consumption for these two substrates when they were oxidized separately. Moreover, respiration of urethan plus succinate occurred twice as rapidly as for each of these compounds alone, which were metabolized at about the same rate. These results suggest that U-grown cells oxidize succinate and urethan simultaneously. Despite the lack of a specific inhibition of succinic dehydrogenase, urethan nevertheless generally decreased by 20 to 59% the rates at which the 2-, 4-, and 6-carbon compounds were respired (table 1). With respect to this, the most marked effects which were observed with succinate and fumarate are considered to be in accord with the reported action of urethan. Whether the carbamate acts directly on the enzymes or otherwise is not known. But these experiments included no dyes which could be effected by the carbamate, as apparently occurred in the Thunberg studies of SHIMAKOSHI (1953).

In other attempts to inhibit succinic dehydrogenase, malonate (20.0 μM), which was itself not appreciably respired by PGY- or U-cells, did not significantly affect the rate or extent of oxidation of succinate by *S. nitrificans*, regardless of the medium on which the actinomycete was grown. In these experiments, it is possible that the cells were impermeable to malonate despite their being frozen before use. Similar difficulties with malonate have been encountered by others (TAKAHASHI and NOMURA, 1952; BARRON and GHIRETTI, 1953).

Summary and conclusions.

The results of the present study indicate that *S. nitrificans* is capable of carrying out reactions which occur in the tricarboxylic acid cycle. This was observed in Warburg experiments with cell material grown on urethan as well as on peptone, yeast extract, and glycerol. However, the findings do not conclusively establish that these reactions constitute the terminal respiratory pathway in *S. nitrificans*. The mere presence of enzymes which mediate the breakdown of certain substances does not necessarily prove the existence of a catabolic mechanism involving these compounds. The quantitative importance of the tricarboxylic acid cycle in *S. nitrificans* would require evaluation on the basis of rates of substrate oxidation compared with the overall respiration, as pointed out by KREBS (1954). Urethan-grown cell material was characterized by lower quotients for both autorespiration and the oxidation of 2-, 4-, and 6-carbon substrates, but oxidized pyruvate and α-ketoglutarate more rapidly than PGY-homogenates. In contrast to this difference, *S. nitrificans* oxidatively assimilated approximately the same percentage of substrates regardless of composition of the medium on which the actinomycete was grown. Neither urethan nor malonate specifically inhibited the succinic dehydrogenase activity of U- or PGY-grown *S. nitrificans*, which in the case of malonate may have been due to permeability obstruction. Among the tricarboxylic acid cyle reactions studied, succinic dehydrogenase and fumarase were the most repressed in mycelium harvested from the urethan medium.

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OBSERVATIONS ON THE USE OF DRIED *SALMONELLA* ANTIGENS

by

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(Received September 18, 1954).

The method of mass production of *Salmonella* antigens described by ANDERSON (1953) led us to further experimental work on the possibility of obtaining these organisms in a dehydrated form. Some notes in the literature (BEGUIN and GRABAR, 1953; ROSCHKA, 1950; HENDERSON and MORGAN, 1938) were encouraging. The latter workers produced highly antigenic extracts from dehydrated *S. typhi* 0-901. On the other hand FELIX (1950) states that "No serologist, who has had experience of the practical problems involved, would now seriously suggest the employment of dried bacteria in the preparation of diagnostic agglutinable suspensions".

As we could not find any publications which discussed the properties of the dried bacteria in detail, we decided in these experiments to examine their agglutinability, absorptive capacity and antigenicity, after rehydration. It soon became evident that the flagellar antigens behaved irregularly and were in most cases almost completely destroyed by this method. Therefore, to avoid any interference from H-antigens, *S. typhi* 0-901 was chosen and, except when otherwise stated, this strain and its antiserum were used in the experiment.

With ANDERSON's technique it was possible to obtain large quantities of antigen in a very pure form without the disadvantages of HENDERSON and MORGAN's technique. Large trays of nutrient agar are occasionally contaminated even when seeded in a sterile room; harvesting is more difficult, and small bits of media are often present in the antigen suspensions.

ANDERSON's method, with a few minor modifications, was therefore adopted. The air-pump was an aquarium type, which would take two or three bottles at the same time. Instead of a large Seitz filter to sterilize the air, a 10 cm glass tube, diameter 1 cm, was filled with non-absorbent cotton wool, sterilized in the autoclave and mounted on top of the aerator as a filter.

The culture was incubated at 36—37° C. for 48 hours with continuous aeration and then harvested. Occasionally a leak in the cellophane sac allowed growth to occur in the medium outside the sac and thus reduced the density of the suspension in the sac.

The suspension was checked for purity and smoothness immediately after harvesting. So far only one bottle has been contaminated and no cultures have turned rough.

The advantage of growing organisms by this method is that it is simple, little work is involved, and relatively small quantities of media are used.

The first objective was to dehydrate the antigen and obtain it in the form of a fine powder.

1. TECHNIQUE OF DRYING THE ANTIGEN.

A variety of methods was tried with some factors in common: a progressive dehydration either with alcohol or acetone, and finally replacement with ether, with evaporation in vacuo by a water-pump.

The most suitable procedure was found to be: Kill the culture with 2—3 volumes of alcohol. Centrifuge at 3000 rpm. for approximately 30 minutes; 2 hours later, decant the supernatant and resuspend in 10—20 volumes of distilled water to remove most of the salt. Centrifuge again for 30 minutes and resuspend the deposit in 5—10 volumes of absolute alcohol. The following day replace the alcohol with acetone. Change the acetone 3 times at 24 hour intervals, replace with ether, connect the flask to a water-pump and evaporate.

By this method a very fine, white, dry powder, easy to resuspend in saline, is obtained.

If the salt is not removed from the suspension, it is often, but not always, difficult to dehydrate the product which may remain in the form of a sticky mass.

The mean yield of one cellophane sac was about 1.5 g of dried powder, occasionally as low as 0.5 g, and sometimes over 2.0 g. This corresponds to the yield from 40—50 8 oz. flat bottles of nutrient agar, or one of HENDERSON and MORGAN's trays (1938).

2. AGGLUTINABILITY.

Three antigen suspensions, containing respectively 100, 50 and 25 mg per 100 ml, were prepared from the powder. When antigen in the following experiments is stated in mg, it will refer to the dehydrated form.

Titrations of those suspensions against international To-standard serum (Bull. Hlth. Org. L.o.N. **7**, 707, 1938; FELIX, 1950) (Colindale, London) with a titre of 1/1,200, gave the following results.

TABLE 1.

Antigen conc. in suspension	Titre against standard serum
A = 1 mg/ml	1/800 ± 1/1600
B = 0.5 mg/ml	1/1600
C = 0.25 mg/ml	1/3200

Suspension A = 1 mg/ml was adopted as a standard antigen for the rest of the experiment. The reaction was of a good quality, being a total agglutination in completely clear fluid. The end point was well defined.

It must be admitted however, that suspension A was concentrated. It corresponds in opacity to Brown's tube 5 = 2289×10^6 bacteria/ml. This concentration is recommended by some workers (MACKIE and MCCARTNEY, 1948) but others (Cours de Microbiologie, Institut Pasteur, 1949—50; GASTINEL, 1949, CALMETTE *et al.*, 1949; BEGUIN, 1953, etc.) prefer a more dilute suspension (500—800×10^6 bacteria/ml), equal to suspension C.

As a control, two suspensions of To antigen A_1 and C_1 (prepared from agar cultures, killed with 3 volumes of alcohol, and resuspended in phenol saline) were made up to the same opacity as suspensions A and C. Judged by agglutination with the standard serum the sensitivity of A_1 was equal to A and C_1 equal to C. This suggests that the antigen made from the powder, and the antigen prepared in the conventional manner are basically the same, and with equal opacity, identical in sensitivity. Once again it must be emphasized that the sensitivity of an antigen suspension should always be adjusted by using an international standard serum to obtain comparable results.

Sera from 75 patients were then titrated with four antigens A,

A_1, C and C_1. Results similar to table 1 were obtained. The less concentrated antigens give the higher titres but the results with rehydrated antigens exactly parallel those with antigens prepared by orthodox methods.

3. Specificity of the rehydrated antigen.

The next question was whether the manufacturing and drying processes had changed the antigenic structure, so that reactions occurred with other sera.

A standard, rehydrated, antigen suspension was titrated against polyvalent somatic sera OA, OB, OC and OD (Kauffmann, 1951). OA was positive, OB, OC and OD negative. Tested against the single radicals of this group, only IX and XII showed reaction, which is in agreement with Jennen's (1954) recent publication.

Furthermore, serum prepared by rabbit inoculation with the rehydrated antigen gave a consistently high titre $\geqslant$ 1/3,200.

Apart from cross reactions with XII containing organisms no antigens gave a titre of over 1/100.

4. Stability of the rehydrated antigen.

Two suspensions were prepared from the powder, the one equal to A, and the other 8 times stronger. Both were stored at 4° C. for six weeks, and during this period, titrated 3 times against the same serum. The concentrated antigen was diluted immediately before use. A_1 was titrated each time as a control. The titres of 1/800 remained from the day of the preparation of the antigen suspensions to the end of the experiment, six weeks later. The rehydrated antigen diluted or concentrated, is stable for at least six weeks.

5. Absorptive capacity.

Numerous experiments were carried out with known quantities of antigen which had been rehydrated, centrifuged and the supernatant fluid discarded. To the deposit was added 3 ml of serum (titre 1/6,400).

It is interesting to note that although 150 mg reduced the titre to 1/100 it took a further 50—100 mg to remove all the agglutinins. It was therefore believed that the saturation of the antigen varied inversely to its concentration. To confirm this a further experiment

TABLE 2.

Absorbed with	Titre after absorption
150 mg	1/100
200 mg	1/50
250 mg	0

was carried out. Three ml of serum of the same titre was absorbed with 50 mg rehydrated antigen. The titre was reduced to 1/800, but on reabsorption of the supernatant with another 50 mg the agglutinins were completely removed.

The rehydrated antigen was thus able to saturate a serum; one single absorption with a large dose being inferior to two absorptions with smaller quantities.

The results were the same whether the absorption took place at 37° C. for 24 hours, or at 37° C. for 2 hours and overnight in the refrigerator at 4° C.

6. ANTIGENICITY.

A suspension containing 2 mg antigen per ml saline was prepared, with 0.5% phenol as a preservative. Several batches of rabbits, with two to four animals in each group, were injected intravenously at 4 days interval (BEGUIN and GRABAR, 1953; ROSCHKA, 1950). Blood was taken for titration before each inoculation. Typical results from such experiments are presented below, titres being given as reciprocals.

Titre	0	200	1600	25000	6400		12800		1600	800		±800	I
mg antigen	2	4	8										
Days	0	4	8	12	16	20	24	28	32	36	40	44	
mg antigen	1	2	2										
Titre	0	100	1600	3200	6400		3200		1600	800		±800	II

The amounts of antigen injected were large compared with those usually given. In Group I, where a total of 14 mg was given, remarkably high titred sera were obtained. They are comparable to those found by HENDERSON and MORGAN (1938) with antigen extracts of '0 901'.

The maximum titre occurred from 10 to 14 days after the last injection which corresponds to the period recommended by KAUFFMANN (1951) for bleeding the rabbit. Thereafter the titre decreased rapidly, a tendency which is common to immune processes, *viz.*, an inclination to equalisation, independent of early titres, irrespective of the method used.

This however proved to be a potent antigen. One of the four rabbits in Group II died, but none were lost in Group I although they received larger quantities of antigen.

Two further groups of rabbits were immunized with 3 smaller doses, with an interval of 4 and 14 days respectively between the injections. The inocula were 0.05 mg, 0.1 mg, and 0.2 mg, making a total of 0.35 mg per rabbit.

The results are shown in the following tables:

Titre	0	200	1,600	3,200		± 3,200	800		400	III
mg antigen	0.05	0.1	0.2							
Days	0	4	8	12	16	20	24	28	32	

Titre	0		400		±800	400	400	200	IV
mg antigen	0.05		0.1		0.2				
Days	0	7	14	21	28	35	42	49	

The antibody production in group III is very similar in the beginning to group I and II, but the same high titre was not reached. Group IV, which received the same total quantity as group III, reacted more slowly than group III, and the titres obtained were inferior to any other group. No deaths occurred. The weight of the rabbits remained constant throughout the experiment.

The amounts of antigen used in group II give adequate immunisation and satisfactory titres, but it is probable, judging by the results in groups III and IV, that even smaller doses of antigen would be adequate.

It would therefore seem reasonable to choose an antigen dose of 0.5, 1 and 2 mg = total of 3.5 mg, give the injections at 4 to 5 day intervals, and bleed 10 to 12 days after the last injection.

When, for any reason, speed of immunization is necessary, the method used for group I, could be employed: serum taken one week after the injection of a large dose of antigen might prove satisfactory.

A few experiments have been carried out with motile organisms. A 90—100% motile culture planted in a cellophane sac, showed only 1—2% motile forms after the incubation period.

The agglutinability in the corresponding H serum was reduced whereas the antigenicity (rabbit immunization) was retained. Possibly the flagella were broken off by the constant movement of the media (CRAIGIE, 1931).

It was found that the H-agglutinability was completely destroyed by the drying process. The H antigenicity was almost nullified. The O-antigen behaved in the same way as that of 0-901.

One could obtain a pure somatic antigen by boiling the culture harvested from the cellophane sac for half an hour and then drying it.

Further investigations on the H antigens and O antigens from motile cultures are being carried out.

Conclusion and summary.

1) ANDERSON's method of growing *Salmonella* has proved to be simple, time-saving, efficient and inexpensive.
2) The somatic culture suspension had the same agglutinability and antigenicity as a surface culture. The flagellar antigenic properties were inferior to those from a swarm-agar.
3) A method of obtaining the antigen as a fine powder is described.
4) It is shown that the rehydrated antigen:
    a) can be adjusted to the correct concentration for agglutination purposes.

b) is as specific as it was before dehydration.
c) is stable for a period of at least 6 weeks in the rehydrated state.
d) has good absorptive capacity.
e) is a potent antigen for rabbit immunization.

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A SIMPLE TEST FOR USE IN EXPERIMENTS ON THE OCCURRENCE AND THE PREVENTION OF MICROBIAL LIPOLYTIC SPOILAGE IN FOODS

by

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Microbial lipolytic spoilage in foods, although less frequent than deteriorations such as putrefaction or fermentation, may be a great nuisance in food manufacture, especially where foods are concerned, which contain glycerides derived from fatty acids $<C_{14}$. Such esters are hydrolyzed by microbial lipases to yield free fatty acids like butyric, caproic, caprylic and capric, well-known for their offensive smell as such and unpleasant taste when in salt form. Moreover such fatty acids tend to a β-oxidation, yielding lower methylketones and thus giving rise to perfume rancidity (6). Foods especially vulnerable for such spoilage are butter and margarine, or commodities like substitute almond paste, prepared from coconut or palm kernel oils.

Since it is impossible to prevent the entrance of all potentially harmful, lipolytic micro-organisms during processing or storage of these products, or to inhibit their metabolism by proper reduction of the equilibrium relative humidity (7), many preservatives have been advocated for inhibiting such microbial processes. However, the correlation between antimicrobial potency of the recommended agents determined in fat-free culture media and the results obtained in practice, has been rather poor in a few cases. This is due to two groups of factors, *viz.*, (a) some recommended agents contain an active anionic group and therefore have an antimicrobial action, which is more or less dependent on the pH of the medium (17, 15); and (b) most of the agents contain lipophilic groups, which may cause their partial extraction from the aqueous phase of the foods concerned, which mostly is the site of microbial activity.

In order to account for such influences a simple test representing the actual situation in spoiling foods has been devised. In this test the food is represented by a nutrient solution in contact with hydrogenated coconut oil, and the infection by an inoculum of the strongly lipolytic strain *Candida lipolytica*, in an initial density of the order of 10^4 cells/ml nutrient medium. Lipolysis is followed by titration of the fatty acids formed in the lipid phase on incubation of the system at 30°C.

Preliminary research.

Following the report by GOLDMAN and RAYMAN (4) it was at first tried to emulsify the nutrient solution in the lipid phase in order to obtain as great a contact-surface as possible. Although very stable emulsions were obtained by dispersing the aqueous phase in the molten fat with the help of a "Turmix" blender and thereupon cooling it quickly to about 5°C., this method in our hands did not give reproducible results.

It was therefore decided to apply the technique, suggested — for qualitative purposes — by EIJKMAN as early as 1901 (3). Whereas EIJKMAN first had some tallow solidify and then covered it with nutrient agar, we found it more convenient to simply cover about 20 ml of nutrient solution with 10 ml of molten hydrogenated coconut oil, cooled to about 45°C.

Experimental technique.

Materials.

The hydrogenated coconut oil had a m.p. = 27°C., ester value of 257 mg KOH/g, iodine number of 1.7 g I/100 g and acid value of 0.07 mg KOH/g.

As a nutrient solution 1% dextrose/0.5% Difco dehydrated yeast extract was used. The various lowered pH_0-values of the solutions were obtained by adding a suitable quantity of 10% tartaric acid solution. Sterilization was carried out in a 16 pint pressure cooker for 15 min/121°C. after 5 min. exhausting (9).

Inoculum and inoculation.

The strain of *Candida lipolytica* used was obtained in July, 1951 from the Yeast Division of the Centraalbureau voor Schimmelcultures at Delft. It was monthly transferred to fresh dextrose/yeast extract/agar since. The inoculum used in the experiments was prepared by subculturing in 1% dextrose/0.5% yeast extract broth for about 36 h/30°C.

Of the nutrient solution 20 ml were transferred to a sterile Petri-dish and inoculated with 0.1 ml of the standard inoculum. Thereupon the solution was covered with 10 ml of molten oil.

Analysis.

After 2 weeks' incubation at 30°C. the system was pasteurized for 1 min/100°C. Of the oil phase 5 g were withdrawn and titrated with 0.1 N alcoholic potassium hydroxyde solution, using phenolphtalein as an indicator.

Preservatives tested.

Three groups of antimicrobial agents have been evaluated with the present test, *viz.*, (a) some esters of gallic acid (16); (b) the sodium salts of two acids, recently proposed as preservatives, i.e. dehydroacetic acid = 3-acetyl, 6 methylpyrandione-2,4 (19, 8, 14) and sorbic acid = 2,4 Δ hexadienic acid (11, 2); and (c) sodium benzoate. It should be underlined that until now only the latter acid is permitted in the Netherlands as an adjunct to margarine in a concentration of 0.2%.

The agents used showed the following properties:

butyl gallate m.p. = 141—142°C.
octyl gallate m.p. = 94— 95°C.
dodecyl gallate m.p. = 96— 97°C.
dehydroacetic acid m.p. = 114—115°C.
sorbic acid m.p. = 135—136°C.
benzoic acid m.p. = 120—121°C.

The esters of gallic acid were dissolved in the nutrient solution by adding stock solutions of the esters in glycerol at such a level that the final concentration of glycerol in the aqueous phase amounted to 10% in all cases.

Results obtained.

The results obtained are recorded in Table 1. They are expressed in AD_{50}, that is the dose of the preservative which reduces the spoilage in the preserved system to the order of 50% of the deterioration, occurring in the unpreserved blank.

TABLE 1.

AD_{50} (lipolysis) of a few preservatives.

Acidity (pH_0)	Agent	AD_{50} (%)
5.0	butyl gallate	0.05
5.0	octyl gallate	<0.005
5.0	dodecyl gallate	>0.1
5.0	sodium dehydroacetate	0.1
7.0	sodium dehydroacetate	0.5
5.0	sodium sorbate	0.01
7.0	sodium sorbate	0.02
5.0	sodium benzoate	0.2
7.0	sodium benzoate	1.0

Discussion.

The results obtained with benzoic acid at the two different pH-values can be considered as an evaluation of the new technique itself. In so far as the inhibitory levels found for this agent are of the same order of magnitude as those arrived at in the classical and more recent reports on this subject (18, 1, 5, 12, 13) it is felt that the technique may be considered as representing the microbial processes actually occurring in the aqueous phase.

Another support for the value of the technique may be found in the fact, that the dependence of the AD_{50}-values from pH as found for dehydroacetic acid is in good agreement with what has been found for other test organisms (10, 14). The same holds true for sorbic acid, although here the data reported by other workers are rather scarce(2).

The ratio of the inhibitive concentrations found for the higher esters of gallic acid, derived from alcohols with varying chain length, does not correspond with the sequence obtained in evaluating the same agents in fat-free media with the *Fusarium avenaceum* mycelium inhibition technique (16). This is due of course, to partial extraction of the esters $\geq C_8$ from the aqueous phase in the test reported in this paper. Here we meet therefore a typical example of the usefulness of the new test.

From a practical point of view it is interesting to notice that concentrations of dodecyl gallate, used for antioxidant purposes (16), do not confer any anti-microbial-lipolytic properties to the fatty foods so treated.

Summary.

A simple model test for microbial lipolytic spoilage in foods has been developed, wherein both pH-changes in the aqueous phase and partition equilibria between lipid and aqueous phase is accounted for. The lipid phase is represented by hydrogenated coconut oil; the aqueous phase by a dextrose/yeast-extract/nutrient solution — containing 10% glycerol when testing esters of gallic acid — and the infection by 10^4 cells/ml of *Candida lipolytica*. Lipolysis is followed by titration of the free fatty acids formed.

In this test butyl gallate, octyl gallate, dehydroacetic acid, sorbic acid and benzoic acid turned out to be more or less powerful inhibitors of lipolytic spoilage, whereas dodecyl gallate was found inactive.

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EVALUATION OF A MEDIUM CONTAINING TERGITOL-7 AND TRIPHENYLTETRAZOLIUM CHLORIDE FOR THE DIFFERENTATION OF COLIFORM BACTERIA FROM DRINKING WATER AND FOODS

by

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In 1951 CHAPMAN (2) described a differential medium for coliforms, containing — besides peptone, yeast extract, lactose and bromo thymol blue — tergitol-7 (sodium heptadecylsulphate) as an inhibitor for Gram-positive bacteria (5) and 2.3.5.-triphenyltetrazolium chloride as a colony indicator (3). On this medium („T.T.-agar") *E. coli* was reported to produce greenish-yellow colonies, whereas for other coliforms the colony type was red. Later KULP *et al.* (4) confirmed CHAPMAN's results.

It was then thought worthwile to re-evaluate this medium for the colimetric examination of drinking water and foods.

Tests with drinking water.

Materials and Methods.

A total of 878 samples of drinking water, originating from water supplies, private wells, etc. was investigated.

Five samples of 10 ml water were transferred to 20 ml bottles, provided with a Durham fermentation-tube. The bottles were filled to completion with double strength McConkey broth (6) and thereupon incubated for $\leqslant$ 2 days/37°C. If acidification or gas formation occurred, a loopful of the contents of the bottles was streaked on Endo-agar (6) and on T.T.-agar. The Endo-agar plates were incubated for 24 h/37°C., the T.T.-plates both for 72 h/37°C. and 72 h/45°C. All colonies developing on both media were further tested for fermentation of dextrose and lactose and for liquefaction of gelatin. The isolates showing the pattern characteristic for coliforms were then rendered pure and run in the IMViC-test (6).

Results and Discussion.

The results obtained are represented in Table 1. In the heading of this table the taxonomic indications represent:

E. coli: IMViC = + + — —
E. coli?: IMViC = — + — —
N.C.C. = Non-coli-coliforms

TABLE 1.

Results of the colimetric examination of 878 samples of drinking water.

Behaviour in enrichment test	Number of samples	Cultural characteristics investigated	Classification of isolates (numbers)				
			E. coli	*E. coli*?	N.C.C.	other types	to-tal
Formation of acid only	132	Endo +[1]	1	2	13		16
		,, —	1	0	115		116
		T.T.A.37°C. +[2]	2	2	14[3]	18	36
		,, 37°C. —	0	0	96		96
		T.T.A.45°C. +	0	0	0	0	0
		,, 45°C. —	0	0	96		96
Formation of acid and gas	81	Endo +	20	2	40		62
		,, —	0	0	19		19
		T.T.A.37°C. +	18	2	45[4]	2	67
		,, 37°C. —	0	0	14		14
		T.T.A.45°C. +	12	0	3[5]	0	15
		,, 45°C. —	0	0	66		66

1) Colonies with metallic sheen; 2) Yellow, dry colonies; 3) 2 strains IMViC: ± + — +; 4) 14 strains IMViC: ± + — +; 5) 2 strains IMViC: + + — +.

We confine ourselves to the coliforms forming acid and gas from lactose in McConkey's medium, since the strains forming acid only, present special taxonomic difficulties.

As far as the value of Endo's medium is concerned, we have to notice that only 20 out of 62 (32%) Endo-positive isolates were true *E. coli*, whereas 40 (65%) were definitely not *E. coli*.

However, among the 19 Endo-negative isolates none were *E. coli*. Hence, though giving numerous false positives, Endo's medium in this investigation gave no false negatives.

T.T.-agar at 37°C. was more or less equivalent to Endo-agar: (a) 18 out of 67 (27%) T.T.-positives being *E. coli*; (b) 47 out of 67 (70%) T.T.-positives being no *E. coli*; (c) none out of 14 T.T.-negative isolates being *E. coli*.

The correlation between the behaviour of isolates on Endo- and T.T.-agar was quite fair: $\frac{14}{19}$ — $\frac{62}{67}$, or 74—92%

T.T.-agar at 45°C. was definitely better than the same medium at 37°C. Here 12 out of 15 T.T.-positive isolates (80%) were *E. coli*, and again none of the T.T.-negatives were *E. coli*. This fully confirms the results reported by BUTTIAUX (1).

Tests with foods.

Materials and Methods.

A total of 267 samples of Dutch and foreign margarine, 56 samples of Dutch and foreign butter, 64 samples of sausage and 102 samples of pasteurized bottled milk, was investigated.

Of the sausage samples 5 × 1 g, of the milk 5 × 1 ml, and in the case of margarine and butter 5 × 1 ml of the serum, rendered at 42 ± 2°C., was enriched in brilliant green/bile/lactose/peptone/water (7) for 48 h/37°C.

If gas formation occurred, a loopful of the contents of the tubes was streaked on Endo-agar and on T.T.-agar. The Endo-plates were incubated 24 h/37°C., the T.T.-plates both 72 h/37°C. and 72 h/24°C. Incubation at 44—45°C. appeared to be of no use, since not a single of the coliform isolates did show growth at this temperature range.

Since our experience with coliforms, isolated from water (vide supra), had indicated that Endo- or T.T.-negative isolates never were *E.coli*, only the both Endo-positive and T.T.-positive isolates were investigated. After securing these strains in pure culture they were further analyzed for the IMViC-pattern.

Results and Discussion.

The results have been collected in table 2. It should be remarked that in all cases with gas formation in the enrichment medium microscopic examination of the sediments revealed the prevalence of pleiomorphic, small, Gram-negative rods. In no case lactose-heterofermentative Bacilli in the absence of coliforms interfered with the test. For further confirmation brilliant green/bile/lactose/peptone/water was heavily inoculated with nine strains of *B. macerans* and *B. polymyxa* and incubated for 72 h/37°C.; in no case gas formation or even growth occurred.

TABLE 2.

Results of the colimetric examination of 489 samples of food.

Material	Number of samples	B B L P-1) positive samples (%)	In the B B L P - positive samples2)				True fecal pollution3) (%)
			E+/T+ (%)	E—/T— (%)	E+/T— (%)	E—/T+ (%)	
Butter	56	59	91	6	0	3	4
Margarine	267	52	39	35	19	7	2
Milk, pasteurized and bottled	102	1	0	100	0	0	0
Sausage	64	63	43	20	17	20	2

1) B B L P = brilliant green/bile/lactose/peptone/water; 2) E + = growth on Endo-agar with metallic sheen; T + = growth on T.T.-agar as dry, yellow colonies; 3) True fecal pollution (%) calculated as 8% of E +/T + isolates.

Of the Endo-positive/T.T.-positive strains only 8% was *E. coli*; 15% were indole-negative *E. coli* and 77% showed the IMViC-pattern: — + — +; Aerobacters were not detected.

The correlation between the behaviour of coliforms, isolated from foods, on Endo- and on T.T.-agar is satisfactory: in 63—97% of the cases Endo-positive correlated with T.T.-positive and Endo-negative with T.T.-negative. It may be recalled that in the strains, isolated from water, the corresponding figures were 74—92%, i.e. of the same order of magnitude.

With foods again, the percentage of true *E. coli* among Endo-positive/T.T.-positive is strikingly low: 8%, or at the very most 23%, if one is willing to accept anindologenic strains as still belonging to *E. coli*. The figure for true *E. coli*, 8%, is significantly lower for foods than it was for the isolates from drinking water: 27%; though the figure for definitely non-*E. coli* is of the same order in both cases: 77% for foods, 70% for drinking water.

As far as the incidence of true fecal pollution (presence of coliforms of the IMViC type: + + — —) is concerned, the data obtained show that in the foods investigated this level is extremely low. Especially the absence of any fecal *E. coli* in over 100 samples of pasteurized, bottled milk is very reassuring indeed.

General conclusions and summary.

1. Tergitol-7/triphenyltetrazolium chloride/agar (T.T.-agar) at 37°C. is a no more selective medium for *E. coli* than Endo-agar.
2. T.T.-agar at 45°C. is a more selective medium for *E. coli* than the same medium at 37°C.
3. Many of the coliform isolates giving false positives on both Endo- and T.T.-agar at 37°C., are of the IMViC type: ± + — +. More oecological data on these strains are needed.
4. Brilliant green/bile/lactose/peptone/water is an excellent medium for the selective enrichment of coliforms in foods. In about 500 tests never any false positive test (e.g. due to lactose-heterofermentative bacilli) was encountered.
5. The incidence of true fecal pollution in the 489 samples of foods investigated was reassuringly low: $\leqslant$ 4%, with pasteurized, bottled milk even showing 0% in 102 samples.

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LE DIFFICILE PROBLÈME BACTÉRIOLOGIQUE DES SEMI-CONSERVES DE VIANDES EN BOITES

par

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On appelle semi-conserves de viandes, des produits placés dans des boîtes hermétiquement closes mais ne pouvant être soumis à une stérilisation parfaite; le jambon en boîte en est le type le plus répandu. Leur chauffage à des températures élevées déterminerait des modifications considérables de goût, couleur et aspect; pour éviter ou détruire les germes contaminants il faut donc mettre en oeuvre des mesures prophylactiques ou curatives n'ayant malheureusement pas toujours une efficacité absolue. Il serait ridicule de considérer, cependant, les semi-conserves de viandes comme des produits dangereux pour la Santé publique; leur consommation de plus en plus répandue entraîne en réalité peu d'accidents. Mais ceux-ci peuvent survenir; le fabricant doit tout faire pour les éviter et le bactériologiste alimentaire sera son conseiller bien plus que son contrôleur.

Dans les conditions actuelles de fabrication, en France, 20 à 30% des jambons en boîte ne contiennent pas de bactéries revivifiables par les techniques habituelles; 70 à 80% en renferment en nombre plus ou moins grand. Elles peuvent alors être divisées en trois catégories:

1°. bactéries virulentes ou toxinogènes; on les appelle en général ,,pathogènes'';

2°. bactéries ,,saprophytes'' dans le sens médical du terme mais capables d'altérer la viande ou les ingrédients (gélatine, en particulier) adjoints;

3°. microbes dits ,,inactifs'': saprophytiques, ils sont dénués de toute aggressivité pour l'homme, l'animal de laboratoire et la semi-conserve de viande.

Il semble nécessaire tout d'abord de préciser le mode de préparation des jambons en boîte. Ceci permettra de comprendre plus facilement l'origine des souillures microbiennes et leurs possibilités de destruction ou survie au cours de la fabrication.

Le porc abattu, ,,flambé'' et échaudé doit être mis en chambre froide durant 48 heures environ; on obtient ainsi des chairs fermes plus faciles à travailler. Les jambons sont alors prélevés. On y injecte au moyen d'une longue aiguille, une saumure dite ,,blanche'' ou ,,d'injection''; c'est une solution aqueuse concentrée de ClNa additionnée de nitrate de sodium. Les pièces sont ensuite immergées dans des bacs contenant la saumure dite ,,bleue'' ou d'immersion; elle renferme au moins 15% de ClNa, 0,5 à 0,6% de nitrate, parfois du nitrite de sodium dans les pays le tolérant et 2 à 5% de glucose ou saccharose. Durant ce saumurage l'hémoglobine et la myoglobine sont transformées en nitrosohémoglobine; celle-ci, chauffée, donne un nitrosohémochromogène ayant une belle coloration rose ou rouge, si estimée dans les jambons. Ce processus est dû à l'action du nitrite; dans les pays interdisant son emploi direct, ce dernier provient de la dénitrification des nitrates réalisée par les bactéries dont la présence est indispensable, par conséquent, dans les saumures. Elles donnent par ailleurs au jambon son ,,bouquet'' particulier qu'on corse, encore, par addition de condiments: poivre, girofle, baies de genévrier etc. . . Après un séjour plus ou moins long dans ces cuves, le jambon est prêt pour la mise en boîte et la cuisson. De la gélatine alimentaire en poudre est placée au centre de la pièce; des feuilles du même produit sont intercalées entre les parois métalliques et la viande. La boîte sertie, contre-soudée, est soumise à un vide partiel puis pasteurisée ou tyndalisée. Après ce chauffage, elle est refroidie par immersion ou aspersion d'eau fraiche.

Ce sommaire tableau met en évidence, aussitôt, les multiples souillures bactériennes auxquelles peuvent être soumis les jambons. Nous les énumérerons ci-dessous et signalerons les moyens de les éviter.

1°. La qualité de la viande a une importance considérable. Elle doit provenir d'un animal sain, bien nourri et abattu après une suffisante période de repos. Ingram (36) puis Gibbons et Rose (26) ont montré, que la chair des porcs mal nourris ou fatigués a un pH beaucoup plus élevé (6 à 6,6) que celle des mêmes sujets en bonne condition physique (5,3). Les pH bas favorisent la pénétration du sel dans le muscle au cours du saumurage; spontanément ou par ce phénomène, ils s'opposent aux pullulations microbiennes. Il est utile, en outre, de tuer le porc en dehors des périodes de digestion; au cours de celles-ci se produisent souvent des bactériémies transitoires d'origine intestinale. LEGROUX et JERAMEC (45) l'ont signalé et nous l'avons constaté. L'animal, en outre, doit être en parfait état de propreté au moment de l'abat; les matières fécales recouvrant les téguments y persisteront souvent malgré le ,,grillage'' ou l',,échaudage''; les microbes qu'elles contiennent pourront altérer le produit fini: c'est le cas des *Streptococcus* et *Clostridium*.

2°. toutes les manipulations suivantes contamineront la viande si elles sont effectuées dans de mauvaises conditions d'hygiène. Deux causes principales interviennent, ici:

a) la malpropreté des instruments: les couteaux devraient être flambés; les aiguilles d'injection de saumure seront stérilisées comme l'a justement indiqué LERCHE (44).

b) la malpropreté des manipulateurs. Il est pratiquement impossible de supprimer les porteurs de germes dans le personnel des usines mais on connaît leur rôle capital dans la contamination des viandes par les *Salmonella* et Staphylocoques enterotoxiques. Cette question a donné lieu à de nombreux travaux et nous y avons insisté (11); les mesures capables d'y remédier sont parfaitement discutées dans un rapport du ,,Ministry of Food'' britannique (73) mais n'ont été réalisées dans aucun pays. Au cours du Congrès du Froid tropical à Marseille en 1951 et du 15ème Congrès international de Médecine Vétérinaire à Stockholm en 1953, des voeux ont été émis, réclamant des décisions des Pouvoirs publics. SOULIE vient d'y insister à nouveau (66) mais sans grand espoir car l'examen médical systématique des manipulateurs est considéré comme une atteinte à la liberté individuelle. La meilleure façon d'éviter le danger des porteurs de germes est de leur imposer des strictes précautions d'hygiène générale. On doit, dans toute la mesure du possible, se rapprocher de l'asepsie chirurgicale dans les usines de semi-conserves de viande.

3°. la qualité des saumures domine le problème de la propreté bactériologique des jambons. Cette question est trop vaste pour être abordée ici; on peut dire qu'une saumure active et propre contient surtout des *Achromobacter* et des *Micrococcus* dénitrifiants; un produit à pH élevé, possédant un pouvoir oxydo réducteur puissant renferme au contraire de nombreuses autres espèces de bactéries indésirables: *Enterobacteriaceae, Pseudomonadaceae, Bacillaceae* (*Clostridium* en particulier). Tout doit être fait pour les éviter: qualité bactériologique de l'eau et des ingrédients: sel, épices etc . . Mossel (53) a parfaitement exposé l'influence néfaste de ceux-ci. De même les caves de saumurage doivent être maintenues à des températures basses de + 4 à + 5°; l'air doit être pur, exempt de poussières ou spores de champignons. La haute concentration en ClNa des saumures a un rôle sélectif important sur leur flore bactérienne. Elle favorise le développement des espèces halophiles généralement bienfaisantes aux processus de dénitrification mais ne tue pas pour autant toutes les autres. Il est donc préférable de ne pas les y amener; on compte trop souvent sur la teneur élevée en sel des semi-conserves de viandes pour leur assurer une qualité hygiénique suffisante; celle-ci en réalité a un effet stabilisant bien plus que bactéricide.

4°. les boîtes destinées à recevoir les jambons contiennent toujours de nombreuses spores de *Bacillaceae*; on doit donc les désinfecter ou les stériliser si possible. La vapeur sous pression, les rayons U.V. seront utilisés pour y parvenir.

5°. la gélatine est par la quantité, le plus important des ingrédients entrant dans la composition des semi-conserves de viandes. Nous avons observé qu'elle contient parfois un nombre considérable de bactéries d'espèces variées (14, 15) et en particulier des *Streptococcus faecalis*, des *Clostridium* et même des Staphylocoques pathogènes. Les fabricants de gélatines alimentaires devront, à l'avenir, fournir des produits de qualité hygiénique suffisante pour être utilisés dans la préparation des jambons en boîte. Nous avons tenté d'en fixer les normes bactériologiques (14, 15).

6°. c'est finalement la pasteurisation qui sera chargée de détruire le plus grand nombre possible des microbes existant dans le produit à ce stade final de la préparation. Ses effets doivent être discutés; ils dépendent en effet:

a) de la température atteinte au centre de la pièce. Les Autorités américaines exigeaient il y a peu de temps celle de 60°; Niven (56) vient de nous apprendre qu'elles réclament maintenant 66°. Il n'est pas facile d'y arriver et les divers procédés de pasteurisation fournissent à ce sujet des résultats manifestement dissemblables; certains fabricants sont encore fidèles au bain-marie et d'autres à l'étuve à vapeur d'eau; Gisske (28) préconise l'air surchauffé et pulsé. Quoi qu'il en soit, les Usines européennes ne satisfont pas, en général, aux justes exigences américaines; leur pasteurisation est trop souvent insuffisante.

b) de la résistance à la chaleur des bactéries présentes. Cette propriété originelle permet, comme on le sait, de distinguer des germes thermosensibles et d'autres thermorésistants. Mais le problème en bactériologie alimentaire est beaucoup plus compliqué: on sait par exemple que certains *Micrococcus* thermosensibles dans les conditions habituelles, tolérent des températures beaucoup plus élevées en présence de graisse. INGRAM (38) en a isolé un, résistant ainsi 60 minutes à 74°. Certaines conditions physiques augmentent la thermorésistance: les pH bas, par exemple, pour les *Salmonella*, comme l'ont montré ANELLIS, LUBAS et RAYMAN (1) et c'est le cas des jambons dont le pH moyen est de 5,5—6,2. Phénomène plus curieux: certains anaérobies peu thermorésistants sont capables d'augmenter considérablement leur thermorésistance par simple contact avec des souches ou espèces très thermorésistantes. Ce processus particulier d'entraînement aux températures élevées a été mis en évidence pour le couple *Cl. botulinum* — *Pl. caloritolerans* par MEYER et LANG (47); PREVOT, RAYNAUD et TATAKI (61) l'ont signalé pour *Cl. perfringens* mis au contact de *Cl. sporogenes* var. *stavangerensis*. D'après ces derniers auteurs la substance entraînante existe dans le corps microbien et diffuse dans le milieu. Plus récemment, PREVOT, THOUVENOT, PITRE et BRESSOU (62) ont décrit un aspect nouveau de ce phénomène très intéressant pour la Conserverie: un anaérobie thermophile asporulé thermosensible: *Cillobacterium thermophilum*, devient thermorésistant par contact avec la substance même des conserves de patés de viandes.

Ces aspects nouveaux de la thermorésistance des bactéries ne doivent pas faire oublier celui connu depuis longtemps en laiterie: de nombreux microbes habituellement thermosensibles perdent en partie cette qualité par accoutumance à des températures relativement élevées auxquelles elles sont soumises de façon plus ou moins continue; c'est ainsi qu'on trouve dans des pasteurisateurs des *E. coli* ou *Streptococcus faecalis* tolérant respectivement 65 et 72° pendant 10 minutes ou plus. On les rencontre fréquemment dans les Conserveries d'hygiène générale médiocre.

c) du nombre des microbes présents dans la semi-conserve de viande. Plus il est élevé et moins le résultat final est bon. En effet, parmi les formes végétatives ou spores microbiennes, les individus plus thermorésistants sont souvent en minorité dans l'ensemble des représentants d'une même souche ou espèce. Cette particularité explique les mesures aberrantes observées dans les expériences de laboratoires et appelées ,,skips'' par les auteurs américains. Cette minorité dangereuse sera d'autant plus facilement évitée que le nombre des cellules microbiennes ou spores sera plus faible.

Cette brève étude des facteurs intervenant dans l'efficacité de la pasteurisation est loin d'être complète. Elle montre simplement qu'ils sont multiples et encore mal connus. La destruction des bactéries et de leurs spores dépend surtout de la température centrale de la masse; elle doit être la plus élevée possible, dans la mesure où elle n'entraîne pas de modifications appréciables de l'aspect, du goût, de la couleur et de l'odeur de la semi-conserve de viande.

7°. Après chauffage, les boîtes sont refroidies par immersion ou aspersion. Des contaminations nouvelles peuvent se produire à ce moment au niveau des ,,microfuites'' des sertissures métalliques même lorsqu'elles sont recouvertes d'une ,,contre-soudure''. Les bactéries de l'eau de refroidissement sont capables de pénétrer ainsi dans le produit alimentaire. On conseille d'utiliser pour cette pratique des eaux potables mais nous préférons recommander des eaux systématiquement et fortement chlorées; des *Bacillus* ou *Clostridium* telluriques, des *B. paracoli aerogenoïdes* dont la présence est tolérée dans une eau de consommation la rendent, en effet, dangereuse pour son usage en Conserverie. Ce refroidissement des boîtes a été considéré par certains comme suffisamment dangereux pour proposer sa suppression. Cette opinion ne peut pas être retenue; elle favoriserait la persistance d'une température assez élevée dans la viande et la gélatine pour déclencher la croissance des bactéries thermophiles puis mésophiles; il faut au contraire réaliser un refroidissement le plus rapide possible pour éviter celle-ci.

A la fin de toutes ces opérations, les jambons en boîtes peuvent être dépourvus de bactéries ou spores revivifiables (20 à 30% des cas) ou en contenir. Dans cette dernière éventualité, pourtant, celles-ci peuvent être entravées dans leur multiplication ou germination par différents phénomènes discutés ci-dessous:

1°. le jambon possède des caractères physiques ou chimiques pouvant leur être hostiles:

a) le pH habituellement compris entre 5,5 et 6,2 n'a pas d'influence manifeste; il peut retarder, pourtant, la culture de certains anaerobies.

b) la concentration finale d'un jambon en chlorure de sodium varie de 30 à 50 pour 1.000. Ce taux assez élevé peut favoriser le développement des bactéries halophiles; c'est le cas des Staphylococci, de leurs variétés entero-toxiques et autres Micrococci, des *Pseudomonas* du sel ou des ,,vases'' des saumures, d'*Amoebobacter* bien étudiés par PENSO (59). Il peut ne pas entraver la culture ou la germination des spores de certains *Bacillus*; nous avons toujours remarqué, par exemple, que *B. subtilis*, *B. pumilus*, *B. cereus*, *B. megatherium* toléraient parfaitement $75^0/_{00}$ de ClNa; de même il ne s'oppose pas au développement de nombreux *Clostridium*. Ce dernier point mérite une attention particulière; ces germes thermorésistants causent, en effet, la majeure partie des altérations des semi-conserves de viandes; il était donc important de connaître leur comportement en présence des facteurs dits ,,stabilisants''. Pour SCOTT (65) le pouvoir inhibiteur du ClNa sur le développement de *Cl. botulinum* est fonction de la concentration en eau désignée par l'auteur sous le nom de ,,aw'' (activity of water) (64). L',,aw'' d'un jambon est de 0,95 à 0,96. Certains *Cl. botulinum* A et B cultivent dans des milieux dont l',,aw'' est de 0,95 mais toutes les souches n'en sont pas capables. Pour empêcher certainement la croissance de *Cl. botulinum*, il faudrait donc augmenter la concentration en sel, ce qui rendrait le produit immangeable. HALVORSON (29), après POWELL (60) a remarqué que les spores de *Bacillus* germaient rapidement si l'on ajoutait à leur suspension dans l'eau, de l'adenosine et de la L alanine; il a mis en évidence le même phénomène pour les spores de *Cl. roseum*. La germination ainsi rapidement déclenchée est inhibée par addition de 4 à 9 %de ClNa; ceci semble dû au bloquage d'un enzyme, l'alanine racemase. Ici, encore, on notera les quantités relativement importantes de ClNa nécessaires pour obtenir une sécurité suffisante dans la stabilisation microbienne.

BEERENS (4) a étudié la culture de différents anaérobies dans un milieu décrit par nous (17); il s'agit d'une gélatine nitratée contenant un gros fragment de viande, un petit morceau de fer et des concentrations variables de ClNa; fondée sur les principes de la gélatine glucosée de GIBSON et ABDEL-MALEK (27) elle possède en réduction tous les éléments principaux du jambon en boîte et peut être considérée comme un „micro-jambon''. L'influence du ClNa sur la croissance des *Clostridium*, dans ces conditions, est résumée par l'auteur dans le tableau 1.

TABLEAU 1.

Concentration en ClNa (grammes par litre)	10	20	30	40	50	70	5 (Témoin)
Cl. perfringens	+	+	+	+	0	0	+
Cl. sporogenes	+	+	+	+	+	+	+
Cl. bifermentans	+	+	+	+	0	0	+
Cl. histolyticum	+	+	+	+	+	0	+
Pl. tertium	+	+	+	+	0	0	+
Pl. carnis	+	+	+	+	0	0	+
Cl. thermosaccharolyticum	+	+	+	+	0	0	+

+: culture — pas d'inhibition.
0 : pas de culture — inhibition.

D'après lui *Cl. sporogenes* tolère 70‰ de sel; les autres *Clostridium* ne sont inhibés que par 50 ‰ de celui-ci, concentration rarement atteinte dans les semi-conserves de viandes. En conclusion, si le chlorure de sodium possède un pouvoir bactériostatique certain, il n'en est pas moins inactif sur de nombreuses bactéries sporulées thermorésistantes, au taux utilisé dans les produits nous intéressant.

c) les nitrate et nitrite n'ont pas d'influence certaine ou constante sur la croissance des microbes habituellement rencontrés dans les jambons en boîtes. Celui des nitrites est de l'ordre de 30 à 100 ppm; Scott (65) interprétant les expériences de Tarr (69) conclue qu'à 200 ppm il n'est pas suffisant pour prévenir le développement de *Cl. botulinum* et *St. aureus* au pH usuel des semi-conserves de viandes. Le nitrate se trouve dans ces dernières au taux de 1 à 5 pour mille; Jensen (39) remarque que 60 p. 100 des jambons en boîtes ne contenant pas ce produit se putréfient lorsqu'ils sont inoculés avec *Cl. sporogenes*; par contre 22 p. 100 seulement subissent cette altération s'ils en contiennent 1,25 p. 1000. Stumbo, Gross et Vinton (67) n'ont pas confirmé les résultats de Jensen. Beerens (4) de son côté montre que les *Clostridium* étudiés par lui (voir tableau 1) tolérent parfaitement 10 pour mille de nitrate.

Nous voyons donc que les facteurs dits „stabilisants” des jambons n'ont en réalité qu'une action bactériostatique trop incertaine. Certains auteurs ont fait observer, cependant, que leur association pouvait être synergique. On retiendra à ce sujet l'observation de GORET *et al.* devant paraître dans un prochain volume de la Revue de Pathologie Générale et Comparée.

BEERENS (4) a étudié, à cet effet, le rôle des divers constituants du jambon en boîte sur le métabolisme des *Clostridium* gazogènes. Il a montré que la gélatine, la viande, le nitrate, le fer, le glucose avaient une action variable selon les souches et que des interférences pouvaient se produire. Ainsi le glucose exalte la production des gaz par *Cl. perfringens*; certaines variétés de *Cl. histolyticum* habituellement anaerogènes deviennent énormément gazogènes en présence de gélatine; le nitrate inhibe partiellement le pouvoir gazogène de *Cl. sporogenes* mais cette action est neutralisée par le fer. Tout ceci montre la nécessité d'étudier les propriétés physiologiques et enzymologiques des bactéries isolées dans les produits alimentaires sur des milieux dont la structure est la plus voisine possible de celle de ces derniers.

2°. le paragraphe précédent souligne l'absence de sécurité fournie par les facteurs physiques ou chimiques intrinsèques des semi-conserves de viandes, dans le bloquage des bactéries pouvant les souiller. En réalité, la température de stockage est l'élément primordial pour réaliser celui-ci. Aussi, on recommande de conserver tous ces produits à + 4 — 6°C. La majorité des germes sont incapables de se reproduire à cette température, mais il existe des exceptions représentées par les bactéries psychrophiles. Cette question a fait l'objet de rapports et discussions très intéressants à la "Society of Applied Bacteriology" (46). Dans les viandes, nous avons rencontré uniquement des „psychrophiles facultatifs”; capables de se développer aux températures voisines de 0° C., ils le font également et beaucoup plus vite à 20, 30 ou même 37°. C'est le cas des *Proteus* et *Pseudomonas* rencontrés dans les hachis par KIRSCH *et al.* (42), des *Providencia* signalés par nous (16), des *Achromobacter*, des *Flavobacterium* et surtout des *Lactobacillus*. NIVEN (55) puis EVANS et NIVEN (24) ont montré que ces derniers déterminaient le verdissement des saucissons secs; nous avons trouvé des altérations semblables des jambons en boîtes produites par eux (12, 16). Tolérants des pH de 4 à 8, des concentrations en ClNa de $50^0/_{00}$ et en nitrates de $10^0/_{00}$, ces *Lactobacillus* heterofermentatifs se développent aussi abondamment en 6 jours à 4° qu'en 18 heures à 30°. Ils déterminaient un verdissement important de la surface et des espaces intermusculaires des jambons au cours de leur conservation au réfrigérateur.

Il faut remarquer, par ailleurs, que les basses températures de stockage, si elles sont capables d'entraver la culture de nombreux germes autres que les psychrophiles, n'ont généralement pas d'effet bactéricide absolu. HARTSELL (30) l'a montré pour les *Salmonella*, *Shigella* et *Staphylococcus*, même lorsque les aliments les contenant sont portés à — 25° puis conservés à — 9° ou — 18°.

Les considérations développées ci-dessus mettent en évidence les innombrables difficultés rencontrées par les fabricants dans la préparation des semi-conserves de viandes. Pour obtenir un produit „sain'', il est nécessaire de le préserver des contaminations bactériennes avant sa mise en boîte. Les minutieuses précautions d'hygiène depuis l'abat jusqu'à celle-ci seront les seuls garants de la qualité du produit fini. Certains jugeant les difficultés trop grandes ont essayé de les éviter par addition de substances antibiotiques ou antiseptiques. La subtiline ne semble pas avoir donné des résultats appréciables. HIRSCH (32) la dit active seulement contre *Staphylococcus aureus*. La nisine, de même fournit des résultats inconstants: sporulicide pour *Cl. butyricum* elle n'est que sporostatique pour *Cl. sporogenes*. Il est naturellement impossible pour nous d'envisager l'éventuelle incorporation de produits chimiques antiseptiques dans les semi-conserves de viandes; l'Académie de Médecine et le Conseil Supérieur d'Hygiène Publique de France ont toujours été hostiles à ces pratiques; leur position est juste; on la retrouve dans de très nombreux pays. Si la loi existe, elle n'est pas toujours respectée cependant. On dispose actuellement d'antiseptiques dont la perception olfactive ou gustative est impossible; les méthodes chimiques, elles-mêmes, sont insuffisantes pour les dépister. On tend donc à utiliser pour leur détection des tests biologiques; MOSSEL (52) en a décrit un, utilisant les modifications du pouvoir protéolytique d'un *Pseudomonas* en présence de ces substances.

Ces considérations de biologie générale démontrent la malheureuse incertitude persistant dans la fabrication des semi-conserves de viandes, quant à la qualité hygiénique du produit fini. On devrait donc pouvoir compter sur l'examen bactériologique de ce dernier pour décider finalement si un lot préparé est ou non comestible ou dangereux. Hélas! là encore les résultats obtenus ne fournissent pas de renseignements pleinement satisfaisants. En effet, comme l'a justement répété CLARENBURG (21), un jambon en boîte forme une ,,unité'' de fabrication au milieu d'un lot et parmi cent d'entre elles, une seule peut être parfois dangereusement contaminée. CHEFTEL (18) a montré que les lois de la statistique étaient incapables de préciser pour ces semi-conserves, le nombre d'échantillons à prélever dans un lot pour estimer avec rigueur la qualité de celui-ci. Dans ces conditions l'examen bactériologique fournira seulement une vue d'ensemble sur la valeur des techniques de préparation utilisées dans une usine déterminée. Il ne faut pas sous-estimer son intérêt cependant; en dehors des calculs mathématiques de probabilité, l'expérience montre que les mauvais produits proviennent presque toujours des mauvaises usines; dans les bonnes, ils ne sont qu'exception pratiquement négligeable.

Nous ne parlerons pas ici de la méthode d'analyse bactériologique des semi-conserves de viandes. Lors du Symposium international de bactériologie alimentaire tenu à l'Institut Pasteur de Lille en Octobre 1954, HOBBS (34) a indiqué celle utilisée au Central Public Health Laboratory de Londres. RIEVEL (63) celle de l'Institut de Kulmbach; CLARENBURG (21), enfin, a bien voulu signaler les résultats obtenus dans son Service avec les techniques que nous avions précédemment recommandées (6, 7). Variant dans leurs détails, toutes ont en réalité le même but:

a) mettre en évidence les germes pathogènes

b) déceler les bactéries dites ,,saprophytes'' capables d'altérer le produit.

Retenant les indications fournies par ces examens dans les divers pays, le Comité scientifique du Symposium a finalement proposé des normes pour les jambons conservés en boîtes. Nous allons les énoncer et les discuter.

1. Germes intolérables dans le produit fini.

a) *Cl. botulinum.*

Il doit être absent mais on pourra éventuellement le tolérer si les conditions physiques ou chimiques du milieu s'opposent à la production de toxine. Les travaux américains montrent qu'elle est impossible lorsque la concentration en sel (ClNa + eau) est au moins de 6 p. 100 et la conservation assurée à + 5°. Il n'y a plus à notre connaissance de botulisme dû aux semi-conserves de viandes; Meyer et Eddie (48) signalent d'ailleurs que les conserves industrielles préparées aux U.S.A. ont été responsables depuis 1925 d'une seule intoxication due à *Cl. botulinum* E. Théoriquement cependant, la présence de *Cl. botulinum* dans les jambons est toujours possible, car il est un hôte éventuel de l'intestin du porc. Il peut gagner les muscles au cours de la bactériémie digestive (45); il peut également souiller la viande lors d'un dépeçage mal effectué. Quand les spores infectent celle-ci, il sera difficile ou impossible de les détruire; leur thermorésistance ne sera guère affectée par les conditions intrinsèques du jambon en boîte; il suffit pour le comprendre de se reporter au travail de Sugiyama (68); leur végétation ne sera pas entravée par le pH du milieu habituellement compris entre 5,6 et 6,2. Ingram (37) n'a pu vérifier l'assertion de Meyer et Gunnisson; pour lui *Cl. botulinum* est incapable de produire une toxine active au dessous de pH 4,5, mais celui des semi-conserves de viandes est loin d'être aussi bas.

b) *Salmonella.*

On ne saurait les tolérer, naturellement. Des jambons en boîtes les contenant ont produit des intoxications alimentaires collectives. L'exemple le plus démonstratif est fourni par l'observation de Wildman *et al.* (72): sur 59 personnes ayant mangé un tel produit infecté par *S. wien*, 49 ont présenté de la gastro-entérite ou un syndrome paratyphique. Nous avons nous-même isolé *S. typhi murium* dans des semi-conserves de viandes. Les modes de contamination du produit sont de deux ordres:

1°. L'animal est malade ou porteur de germes. De nombreux travaux démontrent la fréquence des *Salmonella* dans les ganglions mésentériques du porc. Nous rappellerons ceux de Clarenburg (20) qui en trouve dans 2,78% des cas en Hollande, de Menszynski (51), de Kraneveld *et al.* (43) qui isole 25 fois des *Salmonella* chez 150 porcs bien portants, de Hemmes (31) relatant des épidémies locales dues à l'absorption de viande de porc porteur de *S. paratyphi* B, de Huisman (35) insistant sur la relative fréquence de *S. typhi murium*, de Felsenfeld *et al.* (25) trouvant 13,5% de *Salmonella* dans les viandes de porc et les nôtres (8) enfin.

2°. les manipulateurs des conserveries sont malades ou porteurs de germes. Nous avons suffisamment insisté sur leur danger pour ne pas y revenir.

Il est malheureusement impossible en pratique courante d'éviter ces deux modes de contamination. Sans examen bactériologique systématique des carcasses, le Vétérinaire contrôleur est incapable de dépister les animaux porteurs chroniques de *Salmonella*; celui-ci est irréalisable le plus souvent. Par ailleurs les législations française et étrangères ne nous permettent pas de dépister les manipulateurs véhiculant ces mêmes bactéries. La pasteurisation, seule, pourra les détruire; il est donc indispensable qu'elle soit effectuée à des températures les plus élevées possibles et nous sommes d'avis de recommander à ce sujet les nouvelles exigences américaines. Les *Salmonella* ayant échappé à la destruction thermique pourront se développer dans le produit fini si sa température de stockage dépasse 12 à 15°. Le pH du milieu n'est pas assez bas pour s'y opposer; KINTNER et MANGEL (41) ont montré qu'il faut des pH de 4,2 ou inférieurs pour les inhiber. La concentration en ClNa est elle aussi inopérante. CLARENBURG (21) a parfaitement rappelé tout ceci. Bien plus, les nitrates du jambon suppriment parfois la propriété de certaines *Enterobacteriaceae* de fermenter les sucres avec production de gaz; TUBIASH (70) l'a démontré pour les *Escherichia*; dans ce cas les boîtes contaminées ne présentent pas le phénomène du bombage et paraissent consommables.

c) *Shigella.*

La présence des *Shigella* est aussi indésirable que celle des *Salmonella*. Une espèce devient dominante en Europe; il s'agit de *Sh. sonneï* plus résistante aux conditions physiques et chimiques d'environnement. Elle est capable de produire des syndromes d'intoxications alimentaires comparables cliniquement à ceux dus aux *Salmonella*; nous avons montré (9) qu'ils représentent 17,8% des formes cliniques d'infection. Les *Shigella* peuvent être amenées dans la semi-conserve:

1°. par les manipulateurs porteurs de germes ou malades

2°. par la viande elle-même.

On croyait jusqu'ici à l'électivité de ces bactéries pour l'espèce humaine et le singe. Nous avons vu avec DEOM et MORTELMANS (23) qu'on pouvait trouver *Sh. sonneï* dans la moelle osseuse du veau. Cette constatation nous incite à en recommander la recherche chez le porc.

Là encore, la pasteurisation bien conduite sera le seul garant de la destruction de ces germes.

d) les Staphylocoques entero-toxiques.

On connaît l'extrême fréquence des intoxications alimentaires de cette origine. Elle est particulièrement élevée aux U.S.A.; MEYER (49) rappelle la statistique de FEIG montrant que les intoxications staphylococciques sont 5 fois plus nombreuses que celles dues aux *Salmonella*. Elle l'est moins en Grande-Bretagne (75), où l'on a noté en 1951 et 1952, 147 cas sur un total de 6.866 d'origines diverses. Les jambons peuvent les produire; la démonstration est fournie par les nombreuses observations signalées dans les Public health reports (74).

Mais il s'agit dans tous ces rapports de jambons „nus"; ceux conservés en boîtes n'ont pas été signalés à notre connaissance dans ce genre d'épidémies. Les semi-conserves de viandes interviennent cependant; BOYER *et al.* (5), NEVOT *et al.* (54) en ont décrites dues aux „rillettes".

C'est avant tout le manipulateur porteur de germes qui contamine la viande. PARKER (58) vient, une fois de plus, de le démontrer: chez 13 d'entre eux il a trouvé sur les mains ou dans le nez de 12, les types bactériophagiques rencontrés dans les produits alimentaires producteurs des intoxications. Nous avons de même décelé des lots de jambons en boîtes contaminés par des ouvriers porteurs de lésions staphylococciques des mains.

Une pasteurisation rigoureuse sera seule capable de détruire le Staphylocoque, car il existe des souches relativement thermorésistantes. S'il échappe à cette opération, un stockage des boîtes à une température trop élevée déclenchera sa prolifération; la concentration en ClNa ne gênera pas ce germe très halophile tolérant parfaitement, par ailleurs, un pH 4 ou même inférieur. Signalons à ce sujet les faits suivants: un aliment contaminé par un Staphylocoque entero-toxique n'est dangereux que par sa toxine; celle-ci peut être préformée dans la viande avant la mise en boîte; l'enterotoxine staphylococcique est thermostable, mais MEYER (49) la dit inactivée plus facilement par des températures basses (30 minutes à 65°) que hautes (10 minutes à 100°); une pasteurisation judicieuse est donc capable de la neutraliser.

e) les Clostridium toxinogènes autres que *Cl. botulinum.*

Il s'agit surtout des *Cl. perfringens* A thermorésistants décrits par HOBBS *et al.* (33). Depuis cette description, leur rôle est signalé de plus en plus souvent dans les intoxications alimentaires; en Angleterre ils ont produit en 1951—1952 (75) 5 pour 100 de celles-ci, la plupart des cas observés étant en rapport avec des viandes de conserve ou préparées extemporanément. OSTERLING (57) signale également leur rôle fréquent en Suède. Nous avons observé des troubles gastro-intestinaux chez des nourrissons ayant ingéré des laits abondamment souillés par des *Cl. perfringens* A (10). Cependant, récemment, DACK *et al.* (22) ont été incapables de reproduire avec ces bactéries des phénomènes toxiques chez des volontaires humains.

Ils peuvent être introduits dans la semi-conserve par une contamination de la viande elle-même; BECK (3) a émis cette hypothèse. Ils peuvent l'être aussi par des saumures malpropres, des insuffisances d'hygiène des manipulations et des porteurs humains. Leurs spores thermorésistantes résisteront généralement à une pasteurisation bien effectuée et les constituants du jambon seront incapables d'entraver leur germination si le stockage des boîtes n'est pas réalisé à des températures basses.

2. Bactéries indésirables, témoins d'un défaut de préparation de la semi-conserve.

Le Comité Scientifique du Symposium de Lille a jugé que les jambons en boîtes ne devaient pas contenir d'*Enterobacteriaceae*, *Achromobacter*, *Flavobacterium*, *Pseudomonas* et Champignons inférieurs. Toutes ces espèces peuvent, en effet, être détruites au cours de la pasteurisation; leur présence est significative d'une mauvaise fabrication. Cette prescription est raisonnable car les *Achromobacter*, les *Flavobacterium*, les *Pseudomonas*, les *Proteus* sont parfois psychrophiles; ils se développeraient abondamment dans le contenu des boîtes lors de leur conservation à + 4°.

3. Microbes devant être absents, car ils peuvent altérer le produit.

a) les *Clostridium* gazogènes, sauf s'ils sont incapables de se développer dans les jambons en boîtes. *Cl. perfringens* et *Cl. sporogenes* sont les plus fréquents et déterminent d'énormes altérations des viandes et gélatines; insensibles à la concentration saline, aux nitrate et nitrite, au pH du jambon, ils se développeront abondamment au cours d'une exposition du produit à des températures parfois peu élevées (15°). Ils sont introduits dans la semi-conserve par des viandes de mauvaise qualité ou souillées par des féces, des saumures malpropres, par l'utilisation d'instruments ou de boîtes insuffisamment désinfectées et également par les gélatines de qualité insuffisante. Leurs spores thermorésistantes résistent à la pasteurisation et sont donc redoutables pour l'avenir des semi-conserves. Ils sont les contaminants les plus fréquents et les plus dangereux, commercialement parlant, de ces dernières. Ils se rencontrent, surtout, dans des Usines dont l'hygiène générale est déficiente.

A côté de ces *Clostridium* très agressifs, il en est d'autres doués d'un pouvoir gazogène dans les milieux habituels de culture mais incapables de se développer ou de manifester l'activité de leurs enzymes dans les conditions réalisées dans une semi-conserve de viande; le pH, la concentration en sel, la teneur en nitrates ou nitrites s'y opposent. Il est donc important pour le bactériologiste chargé du contrôle de ces produits de déceler cette éventuelle inhibition. Nous le faisons en utilisant un milieu appelé „micro-jambon" dont la composition chimique et les caractéristiques sont identiques à celles des jambons en boîtes. Nous en avons parlé antérieurement (17). Naturellement ces *Clostridium* inactifs sont tolérables.

b) les Streptococci du groupe D de Lancefield peuvent donner au produit fini un goût désagréable de beurre rance ou fromage. On doit donc les éviter. De nombreuses discussions ont été soulevées par leur origine. On peut envisager actuellement les suivantes:

On rencontre parfois des *Streptococcus faecalis* typiques. Ils proviennent des ouvriers aux mains malpropres ou des gélatines de qualité insuffisante.

Barnes et Ingram (2) ont remarqué dans de nombreux cas, la présence de Streptococci D, différents de *Str. faecalis* typique par leur propriété de ne pas cultiver sur milieu au tellurite de potassium à 1/2500 et quelques autres particularités. Il s'agit de *Streptococcus faecium*, hôtes fréquents de l'intestin du porc, celui-ci hébergeant, d'ailleurs aussi, du *Str. faecalis*. Dans ce cas, la contamination des jambons est d'origine fécale; on ne saurait trop insister, pour l'éviter, sur l'importance de la propreté des téguments de l'animal avant l'abat et des manipulations suivant celui-ci, en particulier au cours de l'evisceration.

Les Streptococci du groupe D présents dans la boîte au moment de sa fermeture sont très difficiles à supprimer. Ils sont relativement thermorésistants; leur culture n'est pas inhibée par une concentration en ClNa de 65 p. 1000 et tolère de larges variations de pH.

c) certains *Lactobacillus* heterofermentatifs du groupe *brevis* peuvent produire un verdissement des chairs et leur donner un goût aigrelet. Nous les avons découverts dans un lot de semi-conserves altérées et avons parlé d'eux antérieurement. Psychrophiles, tolérants parfaitement les conditions les plus hostiles, ils proviennent le plus souvent des matières fécales du porc ou de ,,nids microbiens'' fréquents dans les chambres froides insuffisamment nettoyées des Usines. Nos statistiques personnelles montrent que ces germes sont des contaminants rares des jambons en boîtes; ils n'en sont pas moins redoutables et doivent être prohibés.

d) il existe, enfin, un groupe de bactéries comprenant des espèces de *Bacillus*, *Micrococcus* et *Corynebacterium* dites ,,denitrificans'', elles dénitrifient très activement les nitrates avec production d'azote et oxyde nitreux. Elles produisent un bombage des boîtes mais ne rendent pas leur contenu dangereux pour l'homme. Les *Micrococcus* et *Corynebacterium denitrificans* peuvent être généralement évités par une pasteurisation soigneuse, bien que certains des premiers cités soient thermorésistants en présence de graisse (38); les spores de *Bacillus* par contre résistent parfaitement au chauffage. Le Comité Scientifique du Symposium de Lille a donc décidé de tolérer ces derniers mais de prohiber les variétés denitrifiantes des *Micrococcus* et *Corynebacterium.*

Nous désirons signaler une erreur d'interprétation faite parfois par les bactériologistes alimentaires au sujet des *Bacillus denitrificans* dont a parfaitement parlé KINGMA BOLTJES (40). VERHOEVEN (71) a étudié leur métabolisme. Il ne faut pas les confondre avec les *B. licheniformis*, *B. polymyxa* ou *B. macerans* capables, eux aussi, de produire des gaz mais à partir des hydrates de carbone. Ces dernières espèces ne sont pas redoutables dans les jambons en boîtes, ces produits contenant seulement de petites quantités de sucres fermentescibles.

4. Bactéries tolérables dans les semi-conserves de viandes.

Toutes celles non signalées précédemment font partie de cette catégorie representée surtout par des *Bacillus* ou *Clostridium* ,,inertes'', c'est à dire non pathogènes, non toxinogènes et incapables d'altérer le produit. On pourrait se demander si certains ne sont pas injustement acceptés. C'est le cas, en particulier, des *B. cereus*, *B. mesentericus*, *B. subtilis* décrits par MEYER (50) et CHRISTIANSEN *et al.* (19) dans des intoxications alimentaires collectives. Nous ne le pensons pas: leur développement est toujours relativement réduit dans les jambons en boîtes et leur rôle est encore discuté et discutable; Dack *et al.* (22) les considèrent, à juste raison probablement, comme incapables de déterminer les syndromes observés par certains auteurs.

Cet exposé justifie, nous le croyons, le titre que nous lui avons choisi. Le problème des semi-conserves de viandes apparaît comme l'un des plus délicats de la bactériologie alimentaire, non par les techniques de leur analyse, mais par l'interprétation de celle-ci.

Ces difficultés sont pour nous l'occasion de répéter ce que nous avons écrit (13): le bactériologiste alimentaire doit posséder de solides connaissances de bactériologie générale mais doit avoir subi une préparation particulière; sans être un technicien averti de l'industrie alimentaire, il devra posséder des connaissances suffisantes sur le mode de préparation des semi-conserves de viandes pour parler du problème nous occupant ici. Ce sera la seule façon pour lui de déceler les temps dangereux de la fabrication où se produisent le plus facilement et le plus souvent les souillures microbiennes; il lui sera alors facile de remonter aisément de la contamination constatée dans le produit fini à son origine. Il y sera guidé parfois par la constatation de ces ,,associations microbiennes'' sur lesquelles Ingram (38) a justement insisté; elles font prévoir l'origine fécale ou hydrique, par exemple, des souillures.

On ne saurait se contenter pour l'étude des semi-conserves de méthodes d'analyse simplifiées; elles peuvent entraîner des erreurs regrettables. Il faut agir en bactériologiste ,,pur'' et conduire son examen jusqu'à l'identification complète des germes isolés. Il faut en outre étudier leur métabolisme dans un milieu dont les caracteristiques sont voisines de celles du produit où ils ont été rencontrès; à cet effet, nous avons mis au point le ,,Micro-Jambon''. Pour conclure au rôle nocif d'une bactérie, il faut, en effet, pouvoir réaliser avec elle, dans ce milieu, des phénomènes analogues à ceux constatés dans l'aliment altéré.

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HUMAN O ERYTHROCYTES IN THE HEMAGGLUTINATION REACTION FOR RHEUMATOID ARTHRITIS

by

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Sera from patients with rheumatoid arthritis often have the ability to agglutinate erythrocytes sensitized with a non-agglutinating dose of anti-erythrocyte serum. This reaction was accidentally discovered by WAALER (1940). ROSE, RAGAN, PEARCE and LIPMAN (1948) pointed out the diagnostic significance of the reaction, hence the name Waaler-Rose-test or Rose-test. The technique of the reaction was improved by HELLER, JACOBSON and KOLODNY (1949).

The reaction is now in general use and is mostly performed in this way, that the serum to be tested is first absorbed with non-sensitized sheep erythrocytes in order to remove the agglutinins against these cells, which are normally present. Serial dilutions are then made of the serum and to these is added a suspension of sheep erythrocytes sensitized with a non-agglutinating amount of rabbit antisheepcell amboceptor (usually 1/2 or 1/3 M.A.D.; M.A.D. = minimal agglutinating dose). After 1 or 2 hours incubation at 37°C. the test tubes are placed in the refrigerator and the reaction is read the following day.

It is not necessary to use sheep erythrocytes and rabbit amboceptors; erythrocytes and homologous amboceptors of other animals are also suitable (PIKE *et al.*, 1949; WAGER, 1950).

WAGER (1950) found that human O erythrocytes sensitized with anti-O-cell rabbit amboceptor could also give a positive reaction. A remarkable fact is that human erythrocytes sensitized with iso-agglutinins give no reaction, nor do group O rhesus positive cells sensitized with rhesus agglutinins or O cells sensitized with absorbed anti-M and N-sera (ROSE *et al.*, 1948; WAALER, 1940).

The use of human O cells has the obvious advantage that the absorption with non-sensitized cells of the sera to be tested can be omitted. VAN LOGHEM-LANGEREIS (1952) began therefore an investigation into the practical value of this modification of the reaction. With 100 sera from patients with clinically established rheumatoid arthritis and with 102 sera from healthy persons reactions were done, both with sheep erythrocytes and with human O erythrocytes. With human O erythrocytes more negative or only weakly positive results were obtained in sera from rheumatoid arthritis patients and more weakly positive aspecific reactions in sera from healthy persons than with sheep erythrocytes. VAN LOGHEM-LANGEREIS concluded for this reason that sheep erythrocytes were to be preferred.

An experiment performed by us at the same time seemed to indicate that human O erythrocytes did give dependable results. A more detailed investigation was therefore started.

MATERIALS AND METHODS.

Human O blood was collected in an equal volume of modified Alsever's solution [1]). The blood cells were centrifuged and washed three times with saline. Rabbits were injected as indicated by WAGER (1950, p. 38), *i.e.* intravenous with 0.5, 1.0 and 2.0 ml of a 5% cell-suspension with an interval of five days. In this way, however, none of the 4 rabbits injected produced a suitable amboceptor; the obtained antisera gave only negative reactions when used for the Rose test. But when the same series of injections was repeated after a month's rest (this month's rest is probably not necessary) a good amboceptor was obtained every time. The agglutination titer of these amboceptors is low and is raised relatively little by the second series of injections, but the agglutination becomes more clear-cut, with larger floccules.

For example: after one series of injections the titer was 1 : 400, amboceptor not active. After the second series the titer was 1 : 650, amboceptor good. No further improvement of the amboceptor occurred when a third series of injections was given after another month.

The hemolysin titer of these amboceptors was very low. With an excess of complement complete lysis was obtained in only one amboceptor in a dilution of 1 : 50. The amount of amboceptor used for sensitizing was always less than the amount causing the first trace of lysis of the blood cells in the presence of complement; inactivation of the sera of patients to remove the complement was therefore superfluous. This proved to be an advantage: fewer low titered aspecific reactions were now found in sera from healthy persons.

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[1]) We wish to thank Mrs. A. BOOT-BOLT of the laboratory of the Blood Transfusion Service for her kind assistance in providing us with blood samples.

When fresh O cells or cells only a few days old were used, sensitization with 1/2 M.A.D. amboceptor was generally too strong and aspecific reactions occurred in sera from healthy persons. Therefore ± 1/3 M.A.D. was used for sensitization. It was soon evident, however, that the age of the O cells used had great influence on their agglutinability. Cells a week old agglutinate markedly less than fresh ones and with longer preservation this capacity continues to decrease gradually. Equal sensitization provided, the titer of the rheumatoid arthritis sera decreases accordingly. A survey of experiments demonstrating this is given in table 1. The O blood was taken 18–5 in modified Alsever's solution. For the sensitization of the blood cells a 2% erythrocyte suspension was mixed with an equal volume of a certain amboceptor dilution, varying between 1/2 and 1/3 of the first agglutination titer. The mixture was placed for two hours in the incubator at 37°C. before use. The agglutination titer of the amboceptor was determined by making a series of dilutions, to each of which was added an equal volume of a 1% cell suspension. The tubes were then placed in a waterbath at 37°C. for two hours, and in the refrigerator overnight. The serum dilution in which small floccules could still be seen after shaking was taken as the titer.

The table does not indicate the process entirely clearly, because along with the decrease in titer of the rheumatoid arthritis serum to 1/4 of the original value over a period of five weeks, the floccules also became much smaller.

Sheep erythrocytes kept in Alsever's solution show this decrease in titer too, but less markedly, while the floccules remain larger.

The sheep blood was collected in modified Alsever's solution on 26 - 5, the blood cells were sensitized during the five weeks of the experiment with the amboceptor dilution 1 : 4000.

For our routine reactions we always use fresh sheep blood, never more than one week old. During this one week no decrease in titer could be observed.

TABLE 1.

Titers of a positive rheumatoid arthritis serum and of a negative serum in the hemagglutination test with human O cells in Alsever's solution and of a positive serum with sheep cells in Alsever's solution during a period of five weeks.

date	amboceptor concentrations used for sensitization of the human O cells						titer of anti-O-cell amboceptor	date	amboceptor concentration used for sensitization of the sheep cells	titer of anti-sheepcell amboceptor
	1 : 2800		1 : 3500		1 : 4200				1 : 4000	
	titer pos. serum R_1	titer neg. serum	titer pos. serum R_1	titer neg. serum	titer pos. serum R_1	titer neg. serum			titer pos. serum R_3	
18–5	1 : 256	1 : 16	1 : 256	1 : 4	1 : 256	1 : 4	1 : 1400	28–5	1 : 256–512	1 : 2400
26–5	1 : 256	1 : 8	1 : 256	1 : 4	1 : 128	—	1 : 1000	4–6	1 : 256	1 : 2100
2–6	1 : 256	1 : 4	1 : 128–256	1 : 4	1 : 128	—	1 : 1000	11–6	1 : 128	1 : 2100
12–6	1 : 128	—	1 : 64	—	1 : 64	—	1 : 900	18–6	1 : 128	1 : 2100
22–6	1 : 64	—	1 : 64	—	1 : 64	—	1 : 900	26–6	1 : 128	1 : 2100
								2–7	1 : 128	1 : 2100

TESTS WITH FROZEN BLOOD CELLS.

The instability of O erythrocytes is a disadvantage in practical use. The sensitizing amboceptor dose has to be controlled constantly. In order to obtain a more stable system it seemed to us worth while to try whether or not frozen blood cells retain their agglutinating properties better. The method of CHAPLIN and MOLLISON (1953) for the storage of small quantities of blood in glycerol-citrate mixtures at —20°C. was used, with this difference that a temperature of —40°C. was used to store the blood. 500 ml human O blood was collected in an equal volume of modified Alsever's solution. After 3 or 4 days at 4°C. the mixture was brought to room temperature, centrifuged and the plasma removed. A mixture of 4 volumes of glycerol and 6 volumes of 5% tri-natrium-citrate was then added slowly and mixed thoroughly with an equal volume of the remaining blood cells. This mixture was then divided into quantities of 2.5 ml and frozen at —40°C. in small tubes. After thawing a small amount of blood cells is found to be hemolysed but in the ten months during which we worked with this blood this amount did not increase much. After 10 months at least 0.9 ml of the original 1.25 ml of packed cells per tube remained. It appeared later that hemolysis was even less when a solution of equal parts glycerol and 6% natrium-citrate was used as a preserving fluid.

After having been thawed and centrifuged the cells are washed with solutions containing decreasing amounts of glycerol, *i.e.* 16%, 8% and 4%, in 3% tri-natrium-citrate and finally three times with 1% saline. A 2% suspension in physiologic salt solution was then made of the blood cells and the reactions were carried out as before The erythrocytes were received 21—9—1953 and this same batch was used for 10 months (Table 2).

It is clear that frozen cells give a much more stable reaction. During approximately 10 weeks the titers of the sera 104 and 188 remained almost constant with the same sensitization of the blood cells. Thereafter sensitization has slowly to be made stronger. This is also indicated by a slow decrease in agglutination titer of the amboceptor. In addition to more constant titers the positive sera showed also more pronounced floccules with frozen cells. The ability of frozen blood cells to hemolyse was unchanged after 7 months.

During the period from 7—12—1953 until 26—4—1954 the Rose test was done as routine test with frozen erythrocytes as well as

TABLE 2.

Titers of positive rheumatoid arthritis sera and of negative sera in the hemagglutination test with frozen blood cells during a period of almost 8 months.

Date	titer ambo-ceptor	dose amboceptor used for sensitization	pos. rheumatoid arthritis sera			neg. sera	
			104	188	mixed serum	184	732
24– 9–53	1 : 900	1 : 2700	1 : 256	1 : 64–128		1 : 4	
1–10–53	1 : 900	,,	1 : 256–512	1 : 64–128		—	
8–10–53	1 : 900	,,	1 : 256–512	1 : 32–64		—?	
15–10–53	1 : 600	,,	1 : 128–256	1 : 64		—	
22–10–53	1 : 600	,,	1 : 256	1 : 64		—	
29–10–53	1 : 800	,,	1 : 256	1 : 64		—?	
5–11–53	1 : 600	,,	1 : 256	1 : 64		—	
12–11–53	1 : 700	,,	1 : 128	1 : 64		—	
18–11–53	1 : 600	,,	1 : 512	1 : 128			1 : 4
26–11–53	1 : 700	,,	1 : 128	1 : 64			—
10–12–53	1 : 600	,,	1 : 256	1 : 64			—
24–12–53	1 : 700	,,	1 : 64	1 : 32			—
18– 1–54	1 : 500	1 : 2000	1 : 256	1 : 16			—
28– 1–54	,,	1 : 1500		1 : 32			—
11– 2–54	,,	1 : 1500		1 : 64			—
1– 3–54	,,	1 : 1500		1 : 32			—
24– 3–54	,,	1 : 1200		1 : 64	1 : 1024		—
15– 4–54	,,	1 : 1200		1 : 64	1 : 512		—
5– 5–54	,,	1 : 1200			1 : 512		—
19– 5–54	1 : 300	1 : 1000			1 : 512		—

in the usual way with sheep erythrocytes with all the sera that were received during that period for serological testing for rheumatic diseases. The O cells were sensitized as indicated in table 2, the sheep cells with 1/2 M.A.D. (these cells were at the most one week old). The reaction with the O cells was done with non-inactivated sera. Altogether 617 sera were tested. Of these 501 gave a negative reaction with both methods (a titer of = 1 : 8 was considered positive); 26 sera had titers higher than 1 : 256, since for the routine tests dilutions were made only up to 1 : 256, the titer difference is not known; 29 sera had the same or about the same titers with both methods, the difference did not exceed a two-fold dilution.

The remaining 61 sera gave a larger titer difference. In this group are also included those sera which gave a negative reaction with

TABLE 3.

Comparison of titers in the hemagglutination test with human O erythrocytes and sheep erythrocytes in relation to the clinical diagnosis.

	number of sera	titer with human O cells	titer with sheep cells	clinical diagnosis
The same or nearly the same titers with both methods	501	—	—	Since no differences were observed with the two methods, the clinical diagnosis is not relevant
	26	≧1 : 256	≧1 : 256	
	29	1 : 8—1 : 256	1 : 8—1 : 256*)	
Higher titers with human O cells	22	1 : 128—=1 : 256	1 : 16—1 : 128**)	rheumatoid arthritis
	2	1 : 128	—	,, ,,
	2	1 : 64	—	,, ,,
	4	1 : 32	—	,, ,,
	1	1 : 16	—	,, ,,
	2	1 : 16	—	glomerulonephritis
	8	1 : 8	—	acute rheumatic fever (6) erythema nodosum (1) vague symptoms (1)
Higher titers with sheep cells	1	1 : 64	1 : 256	rheumatoid arthritis
	1	—	1 : 32	,, ,,
	1	—	1 : 16	,, ,,
	1	—	1 : 8	,, ,,
	1	1 : 8	1 : 128	vague rheumatic pains
	2	—	1 : 32	bronchiectasis acute iritis
	13	—	1 : 8	acute rheumatic fever (2) erythema nodosum (1) psoriasis (1) atypical rheumatic disease (3) rheumatic complaints (1) tuberculosis (1) actinomycosis (1) asthma (1) uncertain diagnosis (2)

*) The titer difference between the reactions with the O cells and with the sheep cells was never more than one two-fold serum dilution.

**) The titer difference between the reactions with the O cells and with the sheep cells was more than a two-fold serum dilution.

one of the methods and had a titer of 1 : 8 with the other. In 41 of these sera the titer was higher with the O cells, in 20 with the sheep cells. Of the first 41 sera 31 were from cases of typical rheumatoid arthritis. As for the other 10 reactions, these included 2 cases of glomerulonephritis, 6 cases of acute rheumatic fever or of relapsing rheumatic fever, 1 case of erythema nodosum and 1 case with vague symptoms.

Clinical symptoms of the 20 cases where the reaction with sheep cells gave a higher titer were as follows: 4 cases of rheumatoid arthritis, 1 case of vague rheumatic pains, 1 case of bronchiectasis, 1 case of acute iritis, 2 cases of acute rheumatic fever, 1 case of erythema nodosum, 1 case of psoriasis, 3 cases of atypical rheumatic disease, 1 case of rheumatic complaints, 1 of tuberculosis, 1 probably of actinomycosis, 1 of asthma, 2 cases with an uncertain diagnosis, but not rheumatic illness (see table 3).

It is clear that the O cells give at least equally good and often better reactions than the sheep cells in typical cases of rheumatoid arthritis and that less atypical reactions occur.

DISCUSSION.

The use of human O erythrocytes for the hemagglutination reaction in rheumatoid arthritis is of technical advantage, because the sera to be tested need not be absorbed for the removal of heterophile antibodies. The agglutination capacity of human O cells conserved in Alsever's solution, however decreases fairly rapidly; this causes instability in the results. Dependable results can be obtained with cells frozen at —40°C. in glycerol-citrate mixtures. The agglutination capacity of such cells will also decrease, but very slowly. The same frozen blood sample has been used by us for more than 9 months. It is sufficient to control the agglutination titer once every fortnight. After some time sensitization will have to be stronger. Two positive sera of known titer and a negative serum must always be titrated together with the routine reactions.

In order to obtain a good amboceptor it is necessary to inject the rabbits with more than one series of three injections. Because the hemolysin titer of the amboceptor is lower than the agglutination titer it is possible to use non-inactivated sera in the routine tests, which results in fewer weakly positive reactions in sera from healthy persons. No influence of the inactivation was seen in the titers of the positive sera. The sensitizing amboceptor dose is usually ± 1/3 M.A.D. We establish this by sensitizing blood cells with amboceptor quantities varying between 1/2 and 1/4 M.A.D. With these we determine the titers of two rheumatoid arthritis sera of known strength and of several negative sera. We use the amboceptor quantity with which the positive sera give the right titer while the negative sera give a titer of at most 1 : 4.

Summary.

1. Human O blood cells, frozen in glycerine-citrate mixtures at —40°C., appeared to be very suitable for the hemagglutination test in rheumatoid arthritis. The same frozen blood sample was used for more than 9 months.

2. Absorbtion of the sera to be tested is then superfluous, which is of technical advantage.
3. It is not necessary to inactivate the sera to be tested, since the hemolysin titer of the amboceptor is lower than the agglutination titer. This decreases the amount of aspecific reactions. It is desirable to use fresh sera.
4. More than three injections are necessary for the preparation of rabbit amboceptor.
5. In cases of established rheumatoid arthritis the reaction with human O cells gave more positive results, often with higher titers, than the reaction with sheep cells, while less non-specific reactions occurred.

Acknowledgement.

I am indebted to Miss J. TER BORG and Miss H. POLMAN for valuable technical assistance.

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SPECIES OF *RHODOTORULA* ISOLATED FROM LARYNGEAL SWABS [1]

by

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(Received September 6, 1954).

During the study of fungi in laryngeal swabs we have isolated three species of the genus *Rhodotorula: Rhodotorula mucilaginosa, Rhodotorula flava* and a new species for which the name *Rhodotorula laryngis* is proposed.

Three cultures of *Rhodotorula mucilaginosa* are isolated from three different swabs. Two of these strains are in good agreement with LODDER and KREEGER-VAN RIJ's description (1952).

The third strain gave some diagnostic difficulties. We first thought that this strain was a new species of *Rhodotorula*, because the auxanografic test gave a negative response to maltose. It was therefore sent to Delft together with two other strains. Dra W. CH. SLOOFF, curator of the Yeast Division of the Centraalbureau voor Schimmelcultures retested the organism in Wickerhams liquid medium and then it proved to be positive for maltose in spite of the fact that she too found the auxanografic test negative for this sugar. She then concluded that it was a strain of *Rhodotorula mucilaginosa*.

The two other strains were also tested in Wickerhams liquid medium. One of them proved to be *Rhodotorula flava* with an enhanced production of red carotenoids.

The last culture could not be identified as any of the known species of *Rhodotorula*. The sugar assimilation test with the five sugars which LODDER and KREEGER-VAN RIJ use, show the same result as for *R. mucilaginosa* (positive for glucose, galactose and saccharose

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[1] The technical assistance of Miss ANNA HELGERUD is gratefully acknowledged.

and maltose, negative for lactose). However, if we also use the auxanografic test for raffinose we find that the new strain is negative for raffinose while *R. mucilaginosa* is positive. Also in the shape and size of the cells the new species differs from *R. mucilaginosa*, since the first species has oval cells (2—3,5) × (3,5—6) μ and the last species has short-oval cells (2,5—5) × (4—7) μ. Nor can it be *Rhodotorula rubra* whose cells are long-oval (2—4) × (6—11) μ. Moreover it is positive in raffinose assimilation in accordance with MACKINNON (1946).

Rhodotorula laryngis nov. spec.

In musto maltato cellulae ovoidae, singulae aut binae (2—3,5) × (3,5—6) μ. Sedimentum et anulus subtilis formantur sed non pellicula. In agaro maltato cellulae ovoideae (2—3,5) × (3,5—7,5) μ singulae aut binae. Cultura (post unum mensem, 20° C.) rosea, nitida margine glabro. Pseudomycelium nullum. Fermentatio nulla. In medio minerali cum glucoso, galactoso, saccharoso, maltoso crescit. Nitras calicus non assimilatur. In medio minerali cum alcohole aethylico non crescit. Arbutinum finditur. Amylum non componitur.

Standard description of *Rhodotorula laryngis:*

Growth in malt extract: After 3 days at 25° C. cells are oval, (2—3,5) × (3,5—6) μ, single or in pairs. After one month at 20° C. a sediment and a ring are formed.

Growth on malt agar: After 3 days at 25° C, cells are oval (2—3,5) × (3,5—7,5) μ single or in pairs. After one month at 20° C. the streak culture is red, smooth, glistening with a smooth margin.

Slide cultures: No pseudomycelium.

Fermentation: Absent.

Sugar assimilation:	Glucose	+	Maltose	+ [1]
	Galactose	+	Lactose	—
	Saccharose	+	Raffinose	—

Assimilation of potassium nitrate: Absent.

Etanol as sole source of carbon: No growth.

Splitting of arbutin: Absent.

Starch test: Negative.

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[1] slow in Wickerhams medium.

DISCUSSION.

In spite of *Rhodotorula*-species not having been pointed out as cause of disease, they have been so frequently found in pathological products, air, water and other sources that these yeasts are well worth a further study.

LODDER and KREEGER-VAN RIJ include in the genus *Rhodotorula* seven species and one variety. PHAFF *et al.* isolated in 1952 three new species from shrimps. Till now there were thus isolated 11 species of this genus and more new species will probably emerge.

NAKAYAMA, MACKINNEY and PHAFF (1954) have recently examined carotenoids in asporogenous yeasts. They found that a number of species of *Cryptococcus* had equal carotenoid content as the typical red species of *Rhodotorula* and they conclude "that a separation of the two genera, on the basis of manifest carotenoid is vague and arbitrary. If a separation is to be maintained between the two genera, it should be based on some other characteristic, possibly on the ability to produce the starch-like compound. This has so far only been found in one strain of a red yeast, though it occurs regularly in the yellow yeasts except in *R. flava*."

In accordance with these authors it should be natural to include the genus *Rhodotorula* in the subfamily *Cryptococcoideae*.

Summary.

Three species of the genus *Rhodotorula* were isolated from laryngeal swabs. A new species, *Rhodotorula laryngis*, is described.

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AN INVESTIGATION INTO A SUITABLE MEDIUM FOR THE CULTIVATION OF *MYCOBACTERIUM TUBERCULOSIS* FROM PATHOLOGICAL MATERIAL IN ROLL-TUBES [1]

by

B. HYLKEMA

with the technical assistance of Miss G. A. KLOETEN

(Received December 30, 1954).

The slide culture method of PRYCE (1941) for tubercle bacteria, as transformed for the sake of an examination in the dark field into our culture test in roll-tubes (HYLKEMA and BEKKER, 1955), implies a closer investigation into the suitable medium for this technic. For the obtaining of micro-colonies in the slide test the nutrients in the usual media for the cultivation of tubercle bacteria can be omitted and the most suitable formula for other constituents, a.o. the electrolytes, is likewise uncertain, since the optima for a heavy growth as stated by several authors (PROSKAUER and BECK, SAUTON, LONG, LOCKEMANN, KIRCHNER, DUBOS) differ considerably. Moreover those investigations concern tubercle bacteria from cultures, which grow as far as we know more rapidly on artificial media than tubercle bacteria on primary isolation from the excreta. The only point unanimously agreed upon is the suitability of asparagine as the amino acid needed and the growth promoting effect of glycerol for the human type.

This investigation aimed at studying by means of the roll-tube technic the growth of colonies of tubercle bacteria on media of different composition and at determination of the effect of the single constituents of the usual media. At the same time the principle formerly generally accepted for pathogenic bacteria, that the composition of the medium (NaCl-content, osmotic pressure, pH) should correspond as fully as possible with that of the animal fluids, had to be checked for tubercle bacteria. That principle was roughly realised by the generally used glycerol broth and by some other media (Dorset, Herrold, Lubenau), but turned out as of no real value, since the tubercle bacteria appeared to grow very well in distilled water with 5% egg yolk, as reported by Besredka, and on semi-synthetic media, the composition of which has little in common with that of blood and serum.

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The starting point was the phenomenon, known from the culturing on beef serum by Koch (1884) as well as from the modern rapid-culture method of Pryce, that tubercle bacteria, soon after the host-parasite relation has been cut, will proliferate and will make typical micro-colonies in the absence of any added amino acid. We commenced by studying the growth on ,,deficient'' media lacking the nitrogen source and other constituents; this growth was compared with the growth on a ,,complete'' medium and we further tried to establish which constituent, when added to the medium, would support a normal growth again. The method of determining the rate of growth consisted in the estimation of the number as well as the size of the colonies in the roll-tube, and in noting the snake- or S-like curled bundles wherein the bacteria occur parallel to each other indicating a perfect growth.

Material and methods.

The material for examination consisted of pus from regional glands or, in some cases, other tuberculous matter derived from guinea pigs, which on post mortem examination had shown tubercles in the organs after having been inoculated into the groin with human material. The material was homogenized with 6% H_2SO_4 in the usual way, washed with phosphate buffer (6.3 g $Na_2HPO_4.12\ H_2O$, 1.0 g KH_2PO_4 per l) or with buffered saline and neutralized. For each medium studied, equal doses of the concentrate, ± 0.3 — 0.5 ml, were pipetted in 5 roll-tubes and spread on the surface of the agar by rolling the tubes a few minutes in the roller apparatus. The number of colonies was estimated as described in our previous paper.

The size of the colonies has been measured by estimating how many colonies cover one radius of the microscopic field, adjusted at a length of 1 mm, which appeared a fairly accurate method, or (and) by estimating the diameter by means of an arbitrary eye-piece scale.

We differentiated during counting the colonies according to their diameter, *viz.*, group O: diameter ± 50 μ, group I: ± 100μ (75—

150μ), group II: ± 200 μ (150—300μ), group III: ± 400 μ (300—600μ), group IV: ± 800μ (600—1200μ), and group V: ± 1600 μ (more than ± 1200μ). The grouping in a way that the diameters increase by geometric progression at the rate of 2, makes the classification of the colonies easy, so there is but a small percentage of false observations. Further, when the volume of the colony increases at a same rate in all directions, each doubling of the diameter corresponds with the product of 3 generations.

When the colonies are on a medium containing serum this number of generations is approximately right. Without serum it is however less than 3, since in that case the colonies are flatter. The number of generations of the colonies of group O cannot be estimated, the more so as clumps of tubercle bacteria of about 50μ may occur in the inoculum and persist in the culture without growth.

This method of determining the total output of a culture in a roll-tube may be used for measuring differences in the rate of growth on two media. These differences are indicated by differences in frequencies of groups I to V, when a sufficiently large number of colonies, 150 or more, has been differentiated as to their diameter. The significance of those differences may be calculated by using the chi-square test.

EXPERIMENTAL RESULTS.

The growth on deficient agars without serum.

In studying the growth on such media we used as material pus from a subcutaneous absces of a guinea pig which had been inoculated with a primary culture on Loewenstein. The concentrate had been divided equally on roll-tubes containing different media. The media consisted of 1.8% agar in: distilled water, distilled water with the separate constituents of nutrient broth, and in the basal medium of Dubos. In Table 1 the results of about two weeks' growth on those media are presented.

The great number of colonies will make clear that the more or less rapid growth of the tubercle bacteria in the roll-tube on otherwise unsuitable media is evidently at the expense of nutrients present in the pathological material. Conditional is a material rich in pus, in which tubercle bacteria have multiplied strongly, obtained by subcutaneous inoculation of a culture. Similar experiments with other strains have given corresponding results. The same strains of tubercle bacteria however, when inoculated as dense culture

TABLE 1.

Number of colonies and percentages in each group in cultivating tuberculous pus on deficient media in roll-tubes.

Media, 1.8% agar in	Total number in Groups I—V	Percentage in each group				
		V 1600μ	IV 800μ and V	III 400μ	II 200μ	I 100μ
A. Distilled water	6660		1.0	30.3	27.5	41.2
B. 1% peptone	18470		0.7	12.3	46.0	40.9
C. 0.5% NaCl	8060		0.02	60.8	26.5	12.5
D. 0.5% NaCl, 1% peptone	10525		0.0	46.2	45.6	8.4
E. Meat infusion	11840		0.4	8.1	31.0	60.7
F. Meat infusion, 0.5% NaCl	17810		2.3	20.9	44.6	32.3
G. Nutrient peptone-broth	10180		15.0	23.2	35.1	26.5
H. Dubos' basal medium	21520	2.0	IV 3.5	16.9	38.1	39.6

3.3 mg/l malachite green and 40.000 E/l penicilline have been added to all media. The agar of A—F was bacto-agar, washed with distilled water, the agar of G and H was the usual agar.

suspensions in a buffer solution would show no growth on these same media or sometimes very slight growth on the peptone broth- and basal medium agar.

Table 1 shows further the occurrence of a small percentage of fairly large colonies on the distilled water agar. Furthermore there is no promotion of growth on 1% peptone (B) as compared with A; 0.5% NaCl (C and D), however, does promote growth. On meat infusion (E) smaller and more compact colonies are produced than on distilled water, which become larger in the presence of NaCl(F).

After inoculation with rich material the production of a fairly abundant culture on the usual nutrient broth agar (G), is somewhat more profuse than on the basal medium agar (H).

This growth on deficient agars points to an urge for multiplication in the tubercle bacteria not to be restrained by lack of food. This growth, however, is apparently abnormal. The micro-colonies do not consist of bundles of bacteria parallel adhering to each other, but, according to the microscopical examination of the preparations and the macroscopical aspect (fig. 1, a, b, e) consist of irregular aggregates of isolated rods. Particularly the colonies on the distilled water agar are most irregular in form (fig. 1 h). By timely inoculation from these micro-colonies normal, virulent cultures may again be obtained, though preparations of the micro-colonies stained according to Ziehl-Neelsen are negative or reveal only sporadically an acid fast rod. It appeared that in that case many tubercle bacteria can be made visible by Gram-staining and decolorization according to Much and can be recognized by the granular form of the rods lying at acute angles to each other. We assume the observed phenomena to precede a degeneration and the increase in multiplication on the agar without serum to resemble a dying bloom. The colonies after reaching their highest point in about 10 days soon fail to produce another culture after subculturing.

The growth of colonies of tubercle bacteria on basal medium serumagar.

In marked contrast with the growth on the deficient media is the growth on a complete one. The composition [1] empirically chosen was a compromise between on the one hand the requirements of transparency of the medium and equalizing of the

TABLE 2.

Mean number and diameter of colonies on basal medium serum agar after inoculation of five roll-tubes with an equal quantity of concentrate, after 1, 2 and 3 weeks.

Time of incubation	Mean number of colonies per tube and coefficient of variation	Percentages of the colonies in each group differentiated according to diameter					
		0	I	II	III	IV	V
one week	many of ± 100 μ and 1 of 300 μ						
two weeks	1849 ± 306 (16.6%)	35	46	18	1.2	0	0
three weeks	3275 ± 93 (2.8%)	0	9	32	47.0	9	3
	The same concentrate diluted 1/10						
one week	1.2 of 50 μ and 0.2 of 100 μ						
two weeks	273 ± 32 (11.7%)	23	53	23	1.5	0	0
three weeks	476 ± 24 (5.0%)	0	9	28	50.0	8	5

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[1] Basal medium serum agar, per 1.000 ml: 7.5 g $Na_2HPO_4 12H_2O$, 0.55 g KH_2PO_4, 0.25 g $MgSO_4 7H_2O$, 3 g sodium citrate, 1 g asparagine, 1 g bacto-casitone, 5 mg $FeNH_4$ citrate, 6 ml glycerol destillatum, 0.5 mg $CaCl_2$, 0.1 mg $ZnSO_4$, 0.1 mg $CuSO_4$, 3 mg malachite green, 16 g (bacto)agar washed in distilled water, 100 ml inactivated beef serum kept a month or longer at 3°C., filtrated by Seitz E.K., distilled water q.s. pH 7.4—7.5.

Mg^{++} and K^+ concentrations to those of serum and on the other Dubos' principle to solve in distilled water only the minimum of what is really needed. For the rest we have maintained Dubos' formula (1947) as fully as possible. It appeared to give a good growth of micro-colonies with the characteristic structure.

Table 2 presents the normal development of the colonies on this basal medium serum agar after inoculation with a rather diluted concentrate. As the colonies greatly differ in size, we conclude to an individual inequivalence of the tubercle bacteria in rate of multiplication. In material less diluted than in our experiments the culture test in the roll-tube shows as a rule within a week already a number of macroscopic colonies of 300-400μ. That number amounts to about 0.1%—2% of the final number of colonies. Accordingly when the total number of colonies is less than a thousand the culture test is as a rule not macroscopically positive after one week. Dilution of the inoculum has the same effect. The moment of the first appearance depends obviously on the presence of those single individuals which can multiply extremely rapidly. This explains also the well known phenomenon that the time of first observable growth depends on the number of tubercle bacteria of the inoculation dose (Corper, 1952, and others).

The growth on N-deficient agar with 10% beef serum.

The addition of 10% beef serum to the agar without amino acids or other nutrients acts as a vital force and gives moreover an impetus for a vigorous growth, which is attended with the formation of typical bundles. This is evident when the addition of 10% serum is compared with the addition of 10% distilled water or physiological saline solution (Table 3).

The citrate together with $MgSO_4$ and Fe does not influence the growth of colonies, neither do these substances in combination with the phosphate buffer, but in the presence of serum they do. In that case the $MgSO_4$ and the iron promote the growth significantly as to the size and probably as to the number of colonies. The number of the largest colonies (II and III) is also significantly increased by addition of the phosphate buffer.

The positive Ziehl-Neelsen preparations as well as the woolly aspect and the relief shadow of the mainly compact colonies evidence the growth in curled bundles (fig. 1, c and f). The serum causes

TABLE 3.

Effect of addition of 10% beef serum to media on the growth in three weeks.

Medium 1.6% washed (bacto) agar in	Addition to the agarmedium: (100 ml on 900 ml)	Mean number 1)	Percentages of colonies in the groups O-V differentiated according to diameter					
			O	I	II	III	IV	V
distilled water	distilled water	± 606	18	75	5	2	0	0
	0.85% saline	± 215	8	82	10	0	0	0
	bovine serum	1040 ± 161 2)	5	38	51	6	0	0
3.0 g sodium citrate, 5 mg FeNH$_4$ citrate, 0,43 g MgSO$_4$7H$_2$O in distilled water /0.9 l	distilled water	± 44						
	0.85% saline	± 88						
	bovine serum	1425 ± 114 2)	4	31	47 3)	18 3)	0	0
idem + 8.9 g Na$_2$HPO$_4$ 12 H$_2$O + 0.55 g KH$_2$PO$_4$	distilled water	± 200						
	0.85% saline	a few						
	bovine serum	1410 ± 225	3	19	54 3)	23 3)	0	0
basalmedium with 10% bovine serum		907 ± 114	0	1	6	38	45	10

1) The colonies on media without serum disintegrate and the weakly adhering conglomerates are drifting. The number of colonies on these media is therefore underestimated and approximate.

2) Difference not significant, $P \simeq 0.1$. 3) Difference significant, $P < 0.01$.

these colonies to last a long time, as appeared when they were subcultured into other media. The rich source of amino acids of the basal medium also in our experiments turned out to be unessential for the development of the micro-colonies with the normal structure, though it causes the colonies to increase considerably in volume and makes the bundles to develop into serpentines that are particularly noticeable in the rims (Table 3 and fig. 1, d and g).

Effect of pH on the growth.

The pH has been checked electrometrically on media of the non-inoculated tubes, simultaneously incubated with the cultures the pH of which was checked with indicator paper. Only exceptionally a slight difference between the cultured and the non-inoculated medium has been found.

The effect of the pH on the growth of the colonies when pathological material containing tubercle bacteria has been inoculated on the basal medium serum agar differing in pH, appears from Tables 4 and 5. We have made use of the well known phenomenon

(Dernby, 1921) that the optimum pH curve becomes a relatively narrow peak on a less favourable medium. By leaving out the phosphate buffer in the basal medium serum agar in our experiments B and C in order that in the metabolism the phosphate of only the inoculum could be disposed of, the profusion of growth could be stunted. In experiment A the optimal zone on the normal basal medium serum agar was narrow without our intermediary. In experiment D the conditions for the growth were less favourable by our starting from a liver with tubercles homogenized with Na_3PO_4 to obtain compact colonies of the bovine type, which normally will spread out innumerable minute confluent colonies.

TABLE 4.

Optimal pH for the growth on basal medium serum agar in one week and in two weeks, and the limits of optimal pH-range.

No.	Type of tubercle bacteria	Optimum pH for growth in 1 week	Optimum pH determined on growth in 2 weeks					
			according to number			according to size		
			limit	opt.	limit	limit	opt.	limit
A	human	7.4	6.8	<7.3	>7.65	7.0	<7.3	>7.5
B	human	7.2	6.3	<7.2	>8.1	6.3	<7.2	>8.1
C	human	7.2	6.3	<7.2	>8.0	6.3	<7.2	>8.0
D	bovine	7.3	6.9	<7.3	>7.5	6.9	<7.3	>7.5

TABLE 5.

Relation of pH to number of colonies (100% = 1260) and to the percentages of different size (groups I to V), when inoculating material containing tubercle bacteria of the human type on basal medium serum agar. Growth after two weeks (table 4, A).

pH	I	II	III	IV	V	Total
6.3—6.5	—	—	—	—	—	—
6.8	11.0	16.0	—	—	—	27.0
7.0	17.0	36.5	39.0	12.0	—	104.5
7.1	3.0	33.0	54.0	10.0	—	100.0
7.2	—	6.5	58.0	33.5	2.5	100.5
7.3	—	5.0	59.5	33.0	2.0	99.5
7.4	1.5	33.0	51.0	8.5	1.5	95.5
7.5	9.0	60.0	25.0	—	—	94.0
7.65	31.5	29.5	2.0	—	—	63.0
7.8—8.1	—	—	—	—	—	—

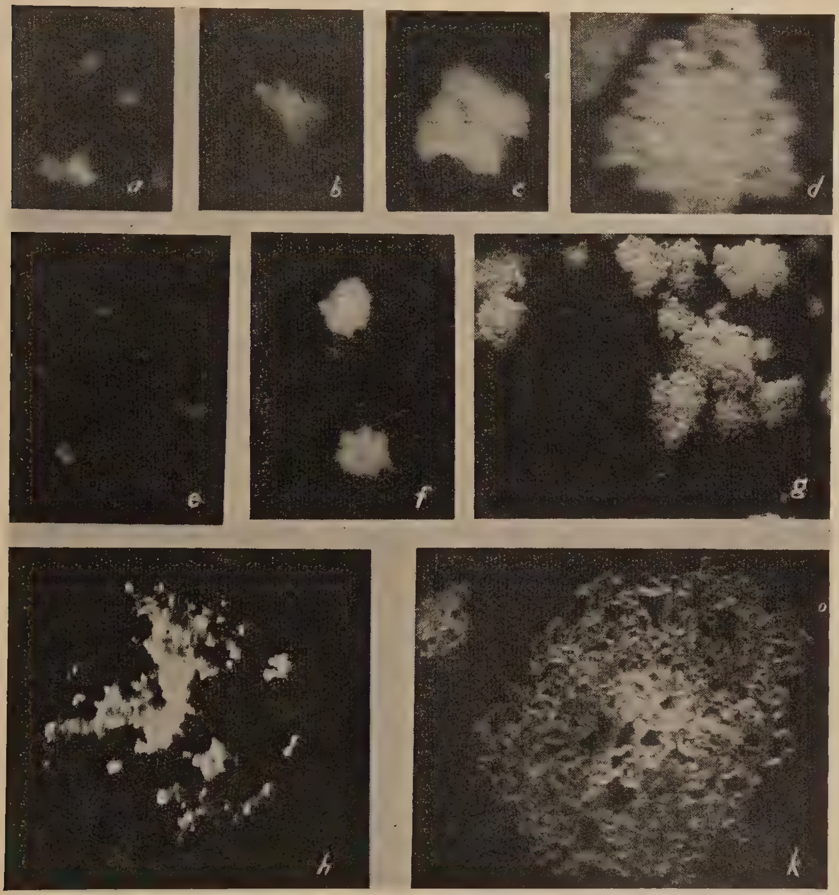

Fig. 1. Colonies in roll-tubes by inoculation of tuberculous material. *a*, *b*, *c* and *d* (32 ×), the same material on distilled water agar with respectively 10% distilled water or saline or serum, and on basal medium serum agar (experiments of table 3). *e*, *f* and *g* (27 ×), colonies of predominant size on resp. distilled water agar, 10% serum agar and basal medium serum agar. *h* (21 ×) large irregular colony on distilled water agar (table 1). *k* (54 ×) colony of bovine type spreading thin serpentines over the wet surface.

For the human as well as for the bovine type the optimum pH for the initial growth during the first two weeks appears to be 7.3, while Table 4 indicates the limits beyond which the differences with the growth at the optimum pH are significant. The advanced prosperous colonies which make the test macroscopically positive in one week, appear only on media with a pH approaching that of animal fluids, 7.2—7.4 (Table 4, column 1). When the serpentines spreading in the rim of the colonies are studied the optimum pH for the initial growth appears to be also of a same value. A more open structure and single snake-like bundles surrounding the colonies of the human type appear with increasing pH. With decreasing pH the colonies become more compact and denser, so that at acid reaction no serpentines can be recognized microscopically. At alkaline reaction, pH 8.0, mother colonies occur which evidently had expelled small S-like bundles which surround the colony.

Discussion.

Estimation of the rate of growth.

The estimation of the diameter of the colonies appeared to be a suitable method, since many factors cause differences in the size without influencing the number of colonies. The mean, theoretical, generation time of a culture, may be calculated approximatively from the percentages of colonies, distributed over the groups I to V. The calculation concerning the growth on the basal medium serum agar may be performed with the figures of table 2 in the following way. The number of generations that has grown after reaching the size of ± 50μ in diameter, amounts on an average after two weeks, to $(46 \times 3 + 18 \times 6 + 1{,}2 \times 9) : 100 = 2.57$; after three weeks to $(9 \times 3 + 32 \times 6 + 47 \times 9 + 9 \times 12 + 3 \times 15) : 100 = 7.95$.

The difference, 5.38 generations, have grown on an average in the third week in 7 × 24 hours. So the time of generation is 31 hours. Youmans and Youmans (1949) calculated for the growth in the basal medium of Dubos with 10% beef serum a mean generation time of 14.4 hours; they took as basis the doubling of the weight of the culture by each generation and the turbidimetrically estimated weights. The difference may be due to a more rapid growth in the fluid medium than on a solid one.

The greater accuracy according to Wilson (1935) in the statistical estimation of the number of colonies by the roll-tube method than by the plating method holds also for tubercle bacteria. When starting 5 replicate tests the standard error was as low as 5—10% of the mean. The heterogeneity of the tubercle bacteria, however, as regards rate of multiplication and susceptibility to inhibitory factors such as we met with in other experiments, does in many cases considerably increase the scatter about the mean value. *Vide* the coefficients of variation of the mean number of colonies after 2 and 3 weeks as stated in Table 2.

The use of roll-tubes is to be preferred to the use of the plating method because of some well known technical advantages (JULIUS, 1938). However, the roll-tube technic is urgently needed for the cultivation of the tubercle bacteria on a relatively large surface, because the film of syneresis liquid which remains intact on the agar in the roll-tubes of the chosen size is indispensable for growth. Fresh agar and an absolute prevention of evaporation are necessary, since a dried surface hinders the growth. The rapid growth of the bovine variety in the roll-tube specially demonstrates the significance of the adhering water, as the bovine variety spreads only in surface liquid as the thin, only microscopically visible, serpentine micro-colonies (fig. 1, k).

The growth of tubercle bacteria on solid media.

The tubercle bacteria which have been inoculated with pathological material on an agar medium without additional source of nitrogen appear to form colonies at the expense of substances present in the material like it is in the slide cultures according to PRYCE. These growth promoting substances may be assumed to be N-containing enzymes, which, as has been demonstrated by SPIEGELMAN (1950) can be used for the building up of nucleoproteins in the absence of an external N-source.

This multiplication continues outside the tuberculous product of the host body, regardless whether the rich amino acids source of the basis medium or less suitable N-containing nutrients are present or not. The N-containing substances all stimulate the growth to a same relatively small extent. Only under the influence of serum, however, does the initial growth start vigorously towards viable colonies, which appear to be dependent on the development of the typical bundles of bacteria in parallel rows characteristic for the tubercle bacteria of the human and bovine variety. The initial growth on a medium containing nutrients gradually changes into the growth that is determined by the specific enzymes for the assimilation of the amino acids resulting in the large colonies on our media. So the cultivation on solid media appears to depend on the cooperation of two different enzyme systems.

For this reason the tubercle bacteria from pathologic material have the advantage over the tubercle bacteria cultivated on artificial media. However, diagnostics will only profit by this advantage when micro-colonies growing under definite conditions and only to be detected microscopically can be examined by a special technic.

The conditions on which the initial growth of tuberculous material on the agar media regardless of the other components depends are the following:

1. A sufficient concentration of certain substances in the material.
2. Presence of serum essential for a vigorous increase in vital bacteria and for the characteristic mode of growing.
3. A wet surface of the agar the fundamental importance of which for the initial growth has been confirmed, since the tubercle bacteria in the roll-tube grow well, even on deficient agars, which otherwise would allow no or minimal growth.

These conditions, each in itself of vital importance for the multiplication are the only essential ones for the primary cultivation of tubercle bacteria on a solid medium.

It has been stated further, that the amount of serum needed in the basis medium agar must be 10% or more and that bovine serum produces better growth than human serum, which KÜRSTEINER (1950) substituted in DUBOS' medium agar for the expensive albumine preparation. We found the albumine solution of lesser value than the bovine serum, a fact in accordance with the extension of the average generation time by 20% as found by YOUMANS and YOUMANS (1949). The effect of the other components of the medium has appeared relatively small when compared with that of the above essential conditions.

The hydrogen ion concentration.

The optimum pH for the cultivation from pathological material should be put at 7.3—7.5. This is on the higher side of the optimum pH zone for the initial growth found after a fortnight as in a further growth the optimum tends to move to the acid side. This is evidenced by the number of colonies reaching equality at the whole pH range from 6.3 to 7.5 and their increasing in size and density when the pH decreases. Moreover the influence of the pH on the structure makes that the serpentines are better recognized at a somewhat higher pH.

This optimum at a level with the pH of the tissue fluids deviates from most optima resulting from tests for the pH which would furnish the largest output after inoculation with cultivated tubercle bacteria. However, the latter optima and the optimum pH for the growth in the third and fourth week, somewhere at pH 6.3 agree perfectly, as might be expected.

The difference with the optimum pH of the initial growth confirms that enzyme systems control the initial growth other than the specific enzymes for the assimilation of amino acids. The latter have evidently an optimum pH at 6.3. We found a same discrepancy between the optima of these two enzyme systems, when a same strain of tubercle bacteria was examined in pus from an inoculated guinea pig as well as from a culture on Loewensten suspended in buffer solution, both grown simultaneously. The pH of the Loewenstein was 7.35, so the difference could not be due to an adaptation to a lower pH of the medium.

The evaluation of media for tubercle bacteria.

Media of various composition compared after having been inoculated with tuberculous material will generally not show conclusive differences (cf. Cummings, 1950). That agrees with the results of the experiments. The growth appears to depend on factors other than the components of the medium, in as far as these are not inhibitory.

The initial appearance of colonies is entirely controlled by variable factors, *viz.*, the concentration of growth promoting substances in the pathologic material, the time the tubercle bacteria need for the formation of the adaptive enzymes, and the distribution of those single rapidly growing bacteria in the material, which as appears from the size and the structure of their colonies dispose of enzymes for the assimilation of amino acids, as if these were constitutive enzymes.

The media which, when inoculated with cultivated bacteria were found to be optimal, showed the large variety in composition, because of the selective adaptation to the media on which these organisms had been cultivated, since the germs that initially grow most rapidly will predominate and determine the properties of the culture.

The results of our investigation may explain the lack of unanimity in the literature regarding the most suitable medium for the cultivation of tubercle bacteria.

Summary.

1. A method is described for the estimation of the growth rate of tubercle bacteria by means of a special roll-tube technic.
2. Rapid and characteristic growth on solid media of tubercle bacteria cultivated from pathological material appears to depend chiefly upon the concentration of growth promoting substances in the material, the addition of serum and the moisture of the surface of the medium. The relatively small effect of other constituents offers an explanation for the lack of unanimity concerning the most suitable medium for the cultivation of tubercle bacteria.
3. Next to the three essential conditions the pH appears to influence greatly the growth of tubercle bacteria. The optimum pH for the initial growth in the primary isolation amounts to 7.3—7.5.

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BACTERIEN-HAEMAGGLUTINATION

IV. UEBER HAEMAGGLUTINATIONSVERSUCHE BEI 105 COLISTÄMMEN UND ADSORPTION DER HAEMAGGLUTININE [1]

von

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(Eingegangen am 29. Dezember 1954).

Nachdem Collier und Jacoeb (1955), Collier, Sue Tsiew und de Miranda (1955) und Collier und de Miranda (1955) die Haemagglutination durch einen Stamm von *Escherichia coli* (Coli 65) ausführlicher untersucht hatten, war es von Interesse zu sehen, wie sich andere Stämme von *E. coli* verhielten. Zu diesem Zweck wurden 113 Stämme aus Faeces des Menschen gezüchtet, von denen 105 als echte *E. coli* anzusprechen waren. Diese wurden in Difco-Bouillon geimpft, und jeweils nach 3, 8 und 15 Tagen wurde ihre haemagglutinierende Fähigkeit gegenüber Blut von Meerschweinchen, Huhn, Mensch und Hammel untersucht.

Die Agglutinationsversuche wurden in sterilen Wassermann-Röhrchen aus Pyrexglas durchgeführt. Das Blut wurde in Natriumcitrat aufgefangen und dreimal gewaschen. Alle Suspensionen und Verdünnungen wurden in physiologischer Kochsalzlösung hergestellt.

Zur Untersuchung der Haemagglutination wurden in einer Reihe von Röhrchen fallende Verdünnungen der Bacteriencultur in Zweierpotenzen in einem Volumen von 0,5 ml hergestellt. Hierzu wurde je 0,5 ml einer 1%igen Suspension der verschiedenen Erythrocyten gebracht. Nach Durchschütteln blieben die Röhrchen bei Zimmertemperatur (22—25°C.) stehen. Nach völliger Sedimentierung der Erythrocyten nach etwa 1½—2 Stunden wurde abgelesen. Das Ergebnis wurde als stark positiv (+++) bezeichnet, wenn die Erythrocyten die Kuppe des Röhrchens als dicker Film bedeckten, als negativ (o), wenn auf dem Boden ein roter Knopf mit scharfem Rande zu sehen war.

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[1] I, II, III Mitteilung: Antonie van Leeuwenhoek **21**, 113, 124, 133, 1955.

Bei den Versuchen über Serum-Hemmung wurde in einer Reihe von Röhrchen das zu untersuchende Serum in einer Menge von 0,25 ml in Zweierpotenzen verdünnt. Hierzu kam je 0,25 ml der Bacteriencultur, die auf die 2-fache, 4-fache usw. agglutinierende Dosis eingestellt worden war. Nach kurzer Bindung wurde das Blut hinzugefügt.

Die Angabe der Verdünnungen erfolgte stets für die Endconcentration im Hinblick auf das Gesamtvolumen. Befanden sich also im ersten Röhrchen 0,5 ml unverdünnte Cultur und 0,5 ml Blutsuspension, so wurde als Bacterienverdünnung ½ angegeben.

Die Ergebnisse der Untersuchung der 105 Stämme von *E. coli* in über 1200 Einzelversuchen bezüglich ihrer haemagglutinierenden Fähigkeit gegenüber Blut von Meerschweinchen, Huhn, Mensch und Hammel sind in der Tabelle 1 zusammengefasst. Zeigte sich überhaupt keine Haemagglutination oder ging diese nur schwach bis zur Verdünnung von 1/8, so wurde in der Tabelle das Resultat mit ,,o'' bezeichnet. Ging die Haemagglutination aber weiter und war sie vor allem deutlich erkennbar, so wurde der reziproke Wert der letzten deutlich haemagglutinierenden Culturverdünnung in die Tabelle eingetragen.

Im Ganzen wurden 52 Stämme als ,,o'' bezeichnet, und von diesen wiesen 22 in keiner Verdünnung mit keiner der vier Blutarten auch nur spurenweise Haemagglutination auf. Bei 30 Stämmen zeigte sich bei der einen oder anderen Blutart sehr schwache Haemagglutination, die aber nie weiter ging als bis zur Verdünnung von 1/8.

Die anderen 53 Stämme agglutinierten mindestens eine der Blutarten in Konzentrationen von mindestens 1/16 deutlich. Je 1 Stamm agglutinierte allein Hühnerblut bzw. Menschenblut, 6 Stämme agglutinierten allein Hammelblut und 15 Stämme allein Meerschweinchenblut. Von den restlichen Stämmen wurden mehrere Blutarten agglutiniert. 10 Stämme agglutinierten alle 4 Blutarten, wie früher Stamm 65.

Es zeigte sich weiterhin, dass bei manchen Stämmen die Agglutinine bereits am 3. Tage stark wirksam waren und dies auch blieben. Mitunter war zwar am 3. Tage Haemagglutination zu sehen, doch verschwand diese bald. In einigen Fällen trat die haemagglutinierende Wirkung erst später auf.

Da in den meisten Fällen von den verschiedenen Stämmen die Versuche mehrfach wiederholt wurden, zeigte sich, dass die in der Tabelle angegebenen Werte leicht variierten, insofern als gelegentlich höhere oder niedrigere Titer erzielt wurden und Agglutinine gegen eine Blutart langsamer oder schneller verschwinden konnten.

TABELLE 1.

Haemagglutination von Meerschweinchen-, Hühner-, Menschen- und Hammelblut durch 3, 8 und 15 Tage alte Bouillonculturen von 105 Stämmen von *E. coli*. Titer als reciproke Werte.

Coli-Stamm Nr.:	Meerschweinchenblut			Hühnerblut			Menschenblut			Hammelblut		
	3 Tage	8 Tage	15 Tage	3 Tage	8 Tage	15 Tage	3 Tage	8 Tage	15 Tage	3 Tage	8 Tage	15 Tage
74 84 94	0	0	16	0	0	0	0	0	0	0	0	0
68	0	0	32	0	0	0	0	0	0	0	0	0
73	0	32	64	0	0	0	0	0	0	0	0	0
17 18 34	0	64	64	0	0	0	0	0	0	0	0	0
30 103	64	0	0	0	0	0	0	0	0	0	0	0
51	16	0	16	0	0	0	0	0	0	0	0	0
19 24	64	64	64	0	0	0	0	0	0	0	0	0
107	64	64	16	0	0	0	0	0	0	0	0	0
76	32	128	256	0	0	0	0	0	0	0	0	0
61	0	0	0	0	0	16	0	0	0	0	0	0
86	0	0	0	0	0	0	0	16	0	0	0	0
63	0	0	0	0	0	0	0	0	0	0	0	16
67	0	0	0	0	0	0	0	0	0	0	16	0
10 37 69 89	0	0	0	0	0	0	0	0	0	16	0	0
55	0	16	0	0	0	32	0	0	0	0	0	0
16	16	128	256	16	64	32	0	0	0	0	0	0
97	64	32	64	0	16	64	0	0	0	0	0	0
108	128	128	256	0	0	128	0	0	0	0	0	0
110	256	256	256	0	0	64	0	0	0	0	0	0
6	512	512	1024	1024	256	0	0	0	0	0	0	0
44	0	16	0	0	0	0	0	16	0	0	0	0
54	16	0	0	0	0	0	0	16	0	0	0	0
87	16	32	64	0	0	0	16	0	0	0	0	0
39	0	16	16	0	16	0	0	0	0	0	256	64
109	512	128	128	64	16	64	0	0	0	32	0	0
35	0	16	32	0	0	0	16	0	32	16	32	16
100	0	16	16	0	0	0	0	0	16	0	16	16
8	32	0	0	0	0	0	32	0	0	64	32	0
33	16	0	0	0	0	0	16	0	0	16	0	0
81	32	0	0	0	0	0	16	0	0	16	0	0
95	16	16	0	0	0	0	16	16	0	16	0	0
104	32	0	16	0	0	0	16	0	32	32	16	16
29	16	0	16	0	0	0	64	0	0	32	0	0
79	32	16	0	16	32	0	32	64	16	0	0	0
13	16	0	32	0	0	16	0	16	0	0	16	0
7	16	64	16	0	16	64	0	64	32	0	64	32
11	16	16	64	16	0	0	16	0	0	32	0	32
43	64	0	16	32	0	0	16	0	16	32	0	0
1	64	32	0	64	0	0	64	32	0	64	64	16
66	64	64	0	32	0	0	32	64	0	64	16	0
88	64	32	16	16	0	0	64	32	16	32	32	0
4	32	64	32	256	32	32	128	16	64	256	16	32
82	64	64	32	32	32	32	64	64	32	128	64	32
31	128	256	256	128	128	128	64	16	16	128	128	16
alter Stamm 65	512	1024	512	256	512	512	128	128	64	256	256	128
52 Stämme	0	0	0	0	0	0	0	0	0	0	0	0

Es konnte auch vorkommen, dass einer der als negativ bezeichneten Stämme einmal in der Verdünnung 1/4 stark agglutinierte. In der Regel aber blieb das Schema der Agglutinationen konstant.

Im Verlaufe der Untersuchung zeigte es sich, dass bei den meisten agglutinierenden Coli-Stämmen die Haemagglutinine fest mit den

Bacterien verbunden waren. Nur bei 3 Stämmen gingen die Haemagglutinine weitgehend in das Medium über, sodass der Titer des von den Bacterien frei centrifugierten Mediums mehr oder weniger mit dem Titer der Vollcultur übereinstimmte. Es handelt sich um die Stämme 6, 65 und 108.

In früheren Versuchen hatte sich gezeigt, dass die im Medium des Stammes 65 vorhandenen Haemagglutinine bei der Adsorption mit einem fremden Stamm von *E. coli* verschwanden, bei der Verwendung eines anderen Coli-Stammes indessen nicht. Die Adsorption schien also abhängig zu sein von der Natur des verwendeten Stammes. Um diese Frage näher zu untersuchen, wurden folgende Versuche angestellt:

Von den Stämmen 6, 65 und 108 wurde das Medium durch starkes Centrifugieren von den Bacterien befreit. Zur Adsorption wurden verschiedene Coli-Stämme benutzt und zwar die gleichen Stämme 6, 65 und 108, ausserdem aber auch andere Stämme, die haemagglutinierende Eigenschaften zeigten, und solche, die keinerlei Haemagglutinine entwickelten. Von je 20 ml Bouilloncultur wurden die Bacterien abcentrifugiert und einmal mit physiologischer Koch-

TABELLE 2.

Adsorption der Meerschweinchen-Haemagglutinine aus dem bacterienfreien Medium der Stämme 6, 65 und 108 von *E. coli*.

Zur Adsorption benutzte Coli-Stämme:	Medium 6	Medium 65	Medium 108
2	+++		+++
3	+++		+++
4	+++	+++	+++
6	0	0	0
14	+++		+++
16	+++	+++	+++
20	+++	+++	+++
21	+++	+++	+++
31	+	+	+
39	+++		+++
65	0	0	0
76	+++	+++	+
79	+++	+++	+++
82	+++	+++	+++
108	0	±	0
110	+++	+++	+++

salzlösung gewaschen. Hierauf wurde der Bodensatz mit 2,0 ml Medium von Stamm 6, 65 oder 108 gemengt und eine Stunde bei 37° und 18 Stunden bei 4° bewahrt. Hierauf wurden die Bacterien abcentrifugiert, und die überstehende Flüssigkeit wurde auf ihre haemagglutinierende Fähigkeit gegenüber Meerschweinchenblut untersucht.

Die bei den verschiedenen Versuchen erhaltenen Resultate sind in der Tabelle 2 zusammengefasst. Es zeigte sich, dass die drei Medien von Stamm 6, 65 und 108 durch die Adsorption mit den Bacterien von 6, 65 und 108 keinen Titerrückgang aufwiesen, nur im Medium 65 zeigte sich nach Vorbehandlung mit Stamm 108 ein sehr leichter Rückgang. Eine deutliche, wenn auch nicht sehr starke Adsorption zeigte sich weiterhin bei allen drei Medien nach Vorbehandlung mit Bacterien von Stamm 31, und bei Medium 108 nach Vorbehandlung mit Stamm 76. In allen anderen Fällen zeigte sich nach Adsorption mit den Bacterien der verschiedenen Stämme ein völliger Schwund aller Agglutinine aus den vorbehandelten Medien.

Die drei Stämme von *E. coli* 6, 65 und 108 zeigen also eine gewisse Verwandschaft darin, dass nicht nur bei allen drei die Haemagglutinine eindeutig in das Medium übergehen, sondern auch darin, dass durch Adsorption ihrer Media mit den analogen Bacterien die Haemagglutinine nicht verschwinden, während nach Adsorption mit anderen Stämmen in den allermeisten Fällen jegliche haemagglutinierende Fähigkeit verloren geht. Wir haben es hier mit einer Erscheinung zu tun, die man vielleicht am zweckmässigsten als gruppenspecifische Nicht-Adsorption bezeichnen könnte.

Bei verschiedenen derartigen Adsorptionsversuchen hatte es sich gezeigt, dass der Titer des haemagglutinierenden Mediums nicht nur gleich blieb, sondern dass er in den meisten Fällen gestiegen war. Es war mit der Möglichkeit zu rechnen, dass vielleicht nach dem einmaligen Waschen noch grössere Mengen der Haemagglutinin an der Hülle der Bacterien zurück geblieben waren, die dann durch das neue Medium eluiert würden. Dies ist aber nicht der Fall, da die zur Adsorption benutzten Bacterien nach dem Abcentrifugieren nicht weniger haemagglutinierend wirkten als vorher.

Die Titersteigerung war aber mitunter sehr deutlich ausgesprochen. Dies geht aus folgendem Versuch hervor: Bacterienfreies

Medium von Stamm 108 mit einem Haemagglutinationstiter von 1/16 wurde mit den drei nicht-adsorbierenden und zwei adsorbierenden Stämmen behandelt. Die benutzten Bacterien waren je einmal bzw. fünfmal gewaschen worden. Die Resultate sind in der Tabelle 3 zusammengestellt. Nach der Vorbehandlung mit den eigenen Bacterien betrug der Haemagglutinationstiter des Mediums 1/8. Der ursprüngliche Titer war also nicht nennenswert beeinflusst. Nach Vorbehandlung mit den beiden Stämmen 6 und 65 zeigte sich indessen eine deutliche Titersteigerung, die in beiden Fällen bei Verwendung von 5-mal gewaschenen Bacterien das 4-fache und bei Verwendung von nur 1-mal gewaschenen Bacterien das 16-fache des ursprünglichen Titers betrug. Nach Adsorption mit den Stämmen Coli 4 und 82 verschwanden alle Haemagglutinine aus dem Medium 108.

TABELLE 3.

Titersteigerung der Meerschweinchen-Haemagglutinine im Medium von *E. coli* 108 nach Adsorption mit 1-mal bzw. 5-mal gewaschenen Bacterien von verschiedenen Stämmen von *E. coli*.

Medium 108 Adsorbiert mit:		Titer:
Coli 6	1-mal gewaschen	1/256
	5-mal gewaschen	1/64
Coli 65	1-mal gewaschen	1/256
	5-mal gewaschen	1/64
Coli 108	1-mal gewaschen	1/8
	5-mal gewaschen	1/8
Coli 4	1-mal gewaschen	>1/2
	5-mal gewaschen	>1/2
Coli 82	1-mal gewaschen	>1/2
	5-mal gewaschen	>1/2
Nicht adsorbierter Medium		1/16

Die gruppenspecifische Nicht-Adsorption zeigte sich stets, wenn die zur Adsorption benutzten Bacterien lebend waren. Es zeigte sich aber, dass durch Erwärmen denaturierte Keime sich anders verhielten.

In diesen Versuchen wurden die Media von Stamm 6, 65 und 108 je mit Bacterien der drei Stämme adsorbiert. Die hierfür verwendeten Bacterien wurden im lebenden Zustande und nach Erwärmen (56°60′, 63°30′, 70°30′ und 80°15′) zugefügt. Nach erfolgter Adsorption wurden die abcentrifugierten Medien auf ihre Haemagglutinationsfähigkeit gegenüber Meerschweinchenblut untersucht. Die Versuche sind in Tabelle 4 zusammengestellt.

TABELLE 4.

Haemagglutination von Meerschweinchenblut durch Media von *E. coli* nach Adsorption durch erwärmte Bacterien der gleichen Stämme.

Behandlung der adsorbierenden Bacterien:	Medium 6 vorbehandelt mit:			Medium 65 vorbehandelt mit:			Medium 108 vorbehandelt mit:		
	Coli 6	Coli 65	Coli 108	Coli 6	Coli 65	Coli 108	Coli 6	Coli 65	Coli 108
nativ	1/32	1/64	1/32	1/128	1/128	1/64	1/64	1/64	
56° 60′	1/16	1/16	1/16	1/32	1/64	1/64	1/32	1/16	1/16
63° 30′	1/4	>1/2	1/32	1/16	1/32	1/32	1/4	1/2	1/8
70° 30′	>1/2	>1/2	>1/2	1/8	1/8	1/16	1/2	1/2	>1/2
80° 15′	>1/2	>1/2	>1/2	1/16	1/16	1/8	>1/2	>1/2	>1/2
Nicht adsorbierte Controlle	1/16			1/64			1/64		

Es ergab sich, dass die nativen Bacterien in keinem Falle Haemagglutinin adsorbiert hatten, und dass auch Erwärmen auf 56° fast stets ohne Einfluss geblieben war. Aber Erwärmen auf 63° zeigte schon in einigen Fällen, dass nunmehr die Bacterien imstande waren, das Haemagglutinin aus dem Medium weitgehend zu entfernen. Ein Erwärmen auf 70° und 80° war imstande, die Bacterien so zu beeinflussen, dass alle drei Stämme aus den Medien 6 und 108 die vorhandenen Haemagglutinine adsorbiert hatten. Bei Medium 65 war nur eine deutliche Senkung des Titers eingetreten.

Die gruppenspecifische Nicht-Adsorption der drei Colistämme wird also durch Denaturieren der Bacterien durch Erwärmen aufgehoben, sodass sich die Keime nunmehr verhalten, wie andere, die Haemagglutinine adsorbierende Stämme.

In weiteren Versuchen wurden die Medien von Stamm 6, 65 und 108 mit lebenden bzw. 30′ auf 80° erwärmten Bacterien der Stämme 2, 3, 4, 14, 20 und 21 adsorbiert. In jedem Fall wurden aus allen drei Medien sowohl durch die nativen als auch durch die erwärmten Keime alle Haemagglutinine entfernt.

Bei der Denaturierung der Bacterien der drei nicht-adsorbierenden Stämme durch Aethylalcohol ergab sich das gleiche Bild, wie nach Erwärmen auf 80°. Durch die auf diese Weise abgetötenen Bacterien wurden aus dem Medium 6 und 108 restlos alleHaemagglutinine entfernt, während im Medium 65 nur eine deutliche Verminderung des Titers zu sehen war. Aehnlich wirkte Methylalcohol, wenn auch nicht so gleichmässig.

Vorbehandlung mit Formalin hatte viel weniger Einfluss auf die adsorbierenden Bacterien. Die drei durch Formol abgetöteten Bacterienstämme konnten aus den Medien 6 und 65 kein Haemagglutinin adsorbieren, sie verhielten sich also wie lebende oder auf 56° erwärmte Keime. Im Medium 108 wurden indessen die Haemagglutinine vermindert, wenn auch nicht völlig entfernt.

Zur Erklärung für die Erscheinung der gruppenspecifischen Nicht-Agglutination wurde zunächst daran gedacht, dass die drei Stämmen 6, 65 und 108 vielleicht serologisch eine Einheit bildeten, während die adsorbierenden Colistämme davon abwichen. Typenspecifische Antisera gegen *E. coli* standen uns nicht zur Verfügung. Da hier die Beschaffung und Haltung von Kaninchen grosse Schwierigkeiten verursacht, konnten wir nur ein Kaninchen und ein Huhn mit dem Stamm 65 (56°60′) immunisieren und zur Kontrolle ein weiteres

TABELLE 5.

Agglutination verschiedener Stämme von *E. coli* durch vier gegen *E. coli* 65 bzw. 4 gerichtete Antisera und vier Normalsera. Reziproke Werte der Titer.

Agglutination mit	Antiserum gegen 65 von		Antiserum gegen 4 von		Normalserum von		Normalserum von	
	Huhn 1	Kanin. 3	Huhn 2	Kanin. 4	Huhn 1	Huhn 2	Kanin. 3	Kanin. 4
Coli 2	400	100	100	<50	<100	<100	<100	<100
Coli 3	1600	400	200	50	<100	<100	<100	<100
Coli 4	100	<50	**800**	**1600**	<100	<100	<100	<100
Coli 6	6400	6400	50	<50	<100	<100	<100	<100
Coli 14	6400	6400	50	50	<100	<100	<100	<100
Coli 20	400	100	100	100	<100	<100	<100	<100
Coli 21	100	100	<50	200	<100	<100	<100	<100
Coli 31	400	1600	3200	400	<100	<100	<100	<100
Coli 65	**51200**	**51200**	400	100	200	<100	<100	<100
Coli 76	12800	3200	100	50	<100	<100	<100	<100
Coli 79	3200	400	50	100	<100	<100	<100	<100
Coli 82	51200	25600	100	50	400	<100	<100	<100
Coli 108	25600	25600	200	200	<100	<100	<100	<100

Kaninchen und ein Huhn mit dem Stamm 4 (56°60′). Die Immunisierung mit Coli 65 lieferte zwei stark wirkende Sera, die mit Coli 4 nur schwach wirkende Sera.

Mit diesen Antiseren wurden die für die Versuche benutzten Colistämme agglutiniert. Als Controlle dienten je 2 Normalsera vom Kaninchen und Huhn. Die Versuche wurden nach 24 Stunden Aufenthalt im Brutschrank abgelesen. Die Resultate sind in Tabelle 5 zusamengefasst.

Sowohl das vom Huhn als auch vom Kaninchen gewonnene Antiserum gegen Coli 65 wirkte eben so stark gegenüber Stamm 108 und sehr deutlich gegen Stamm 6. Dies würde für eine gemeinsame Antigencomponente sprechen. Da aber auch die gut adsorbierenden Stämme 14, 76 und 82 Agglutinationswerte aufweisen, die in der gleichen Grössenordnung liegen, kann ein gemeinsames Antigen nicht die Ursache sein für die gruppenspecifische Nicht-Adsorption. Bei dem vom Huhn gewonnenen Antiserum gegen Coli 4 findet sich ausserdem eine Mitagglutination mit Stamm 65, trotz der Unterschiede in der Adsorptionsfähigkeit.

Um einen weiteren Einblick in die serologische Verwandschaft der verschiedenen adsorbierenden und nicht-adsorbierenden Stämme zu

TABELLE 6.

Agglutination von *E. coli* 65 und Hemmung der Meerschweinchen-Haemagglutination durch *E. coli* 65 mittels Antisera gegen Stamm 65 nach Adsorption durch verschiedene Stämme von *E. coli*. Reziproke Werte der Titer.

Adsorption der Antisera mit Stamm:	Agglutination von *E. coli* 65		Hemmung der Meerschweinchen-Haemagglutination	
	Antiserum gegen 65 vom Huhn	Antiserum gegen 65 v. Kanin.	Antiserum gegen 65 vom Huhn	Antiserum gegen 65 v. Kanin.
Coli 2	128000	128000	800	1600
Coli 3	64000	64000	1600	1600
Coli 4		128000	800	800
Coli 6	64000	64000	800	400
Coli 20	128000	128000	1600	800
Coli 65	2000	<1000	<200	<200
Coli 79	128000	128000	400	800
Coli 108	128000	128000	400	400
Coli 110	64000	32000	400	400
Nativ-Contr.	128000	128000	1600	1600

gewinnen, wurden die beiden gegen Stamm 65 gerichteten Antisera mit verschiedenen Coli-Stämmen stark adsorbiert. Die adsorbierten Sera und das native Serum wurden hinsichtlich ihrer agglutinierenden Wirkung gegenüber Stamm 65 untersucht. Die Ergebnisse finden sich in Tabelle 6 zusammengestellt.

Während die beiden nativen Antisera je in der Verdünnung von 1/128000 den Stamm 65 agglutinierten, war nach Adsorption mit Stamm 65 der Titer auf 1/2000 bzw. >1/1000 gesunken. Demgegenüber war durch Vorbehandlung mit den beiden zur gleichen, die Haemagglutinine nicht adsorbierenden Gruppe gehörigen Stämmen 6 und 108 keine Titerverminderung eingetreten, ebensowenig aber auch durch die anderen benutzten Colistämme. Die serologische Verwandschaft zwischen Stamm 65 mit Stamm 6 und 108 ist also nicht weitgehend. Auf keinen Fall aber würde serologische Verwandschaft die gruppenspecifische Nicht-Agglutination erklären können.

In der gleichen Tabelle sind ausserdem Versuche aufgenommen, in denen die haemagglutinationshemmende Wirkung der beiden gegen Stamm 65 gerichteten Antisera vor und nach Adsorption mit den verschiedenen Colistämmen festgestellt wurde. Beide Antisera hemmten im nativen Zustand die durch Coli 65 verursachte Agglutination von Meerschweinchenblut in der Verdünnung von 1/1600. Durch Vorbehandlung mit dem homologen Stamm wurde der Titer bis auf >1/200 gesenkt, während durch Vorbehandlung mit den anderen Stämmen allenfalls eine vierfache Titersenkung erreicht wurde. Auch diese Versuche sprechen gegen eine serologische Identität zwischen den Stämmen 6, 65 und 108.

Die haemagglutinationshemmende Wirkung der gegen Stamm 65 gerichteten Antisera, wie sie in Tabelle 6 deutlich wird, erscheint ausserordentlich niedrig, wenn man sie beispielsweise mit den stark hemmenden Effecten vergleicht, die specifische Antisera gegen die Influenzavirus- und Pockenvirushaemagglutinationen auszuüben vermögen. Es sei daher noch folgender Versuch mitgeteilt:

Die vier gegen Stamm 65 und 4 gerichteten Antisera und je zwei Normalsera von Huhn und Kaninchen wurden auf ihre hemmende Wirkung gegenüber der Haemagglutination von Meerschweinchen- und Hühnerblut durch verschiedene Colistämme untersucht. Benutzt wurde das 2-fache und 4-fache Multiplum der einfachen haemagglutinierenden Dosis.

Wie aus der Tabelle 7 hervorgeht, ergibt sich ein sehr buntes

TABELLE 7.

Hemmung der Haemagglutination von Meerschweinchen- und Hühnerblut durch *E. coli* mittels Coli-Immunsera und Normalsera von Huhn und Kaninchen. Reziproke Werte der Titer.

E. coli Stamm:	Multiplum:	Immunsera:				Normalsera:			
		Huhn-Anti-65	Huhn-Anti-4	Kan.-Anti-65	Kan.-Anti-4	Huhn Nr. 1	Huhn Nr. 2	Kan. Nr. 3	Kan. Nr. 4
gegen Meerschweinchenblut:									
6	2×	2560	2560	2560	2560	2560	2560	2560	2560
	4×	1280	640	1280	<40	640	640	40	<40
65	2×	1280	160	640	<40	<40	<40	<40	<40
	4×	160	<40	320	<40	<40	<40	<40	<40
4	2×	<40	<40	640	640	160	80	1280	1280
	4×	<40	<40	160	160	<40	<40	80	80
108	2×	1280	<40	320	<40	<40	<40	<40	<40
	4×	160	<40	160	<40	<40	<40	<40	<40
gegen Hühnerblut:									
65	2×	2560	640	2560	320	320	1280	160	320
	4×	2560	320	2560	80	160	80	40	40
4	2×	80	80	640	1280	160	80	640	640
	4×	<40	<40	320	640	80	40	320	640
108	2×	2560	640	2560	640	2560	160	80	80
	4×	2560	320	2560	160	160	320	<40	40

Bild, das keinerlei System erkennen lässt. So sind beispielsweise die Normalsera unwirksam gegenüber der Haemagglutination von Meerschweinchenblut durch Stamm 65 und 108, während sie bei der Haemagglutination von Hühnerblut in verschiedener Stärke wirksam sind. Das Normalserum von Huhn 1 hemmt beispielsweise die Haemagglutination durch Stamm 108 noch in der Verdünnung von 1/2560, eben so stark also, wie die beiden gegen Stamm 65 gerichteten Antisera. Weiterhin ist interessant, dass die Haemagglutination von Meerschweinchenblut durch Stamm 6 in der 2-fach wirksamen Dosis sowohl durch die vier Antisera, als auch durch die vier Normalsera in der gleichen Concentration von 1/2560 gehemmt wird, während bei der Benutzung der 4-fach agglutinierenden Dosis deutliche Unterschiede hervortreten. Principielle Unterschiede zwischen den Antiseren und Normalseren bestehen nicht. Die Antisera enthalten wohl eindeutig Antistoffe gegen die Bacterien, viel weniger ausgesprochen aber gegen die Haemagglutinine. Analog enthalten auch die Normalsera Hemmungsstoffe gegen die Haemagglutinine, nicht aber gegen die Bacterienagglutinine.

Schliesslich seien noch zwei Versuche mit geteilt, in denen versucht wurde, aus den Medien von Stamm 6, 65 und 108 die Haemagglutinine durch verschiedene Blutarten zu adsorbieren. Im ersten Versuch wurden die drei Medien mit Meerschweinchen-, Hühner-, Menschen- und Hammelblut je im Verhältnis 1 : 1, 1 : 2 und 1 : 10 adsorbiert, im zweiten Versuch wurde nur das Medium von Stamm 65 dreimal hintereinander im Verhältnis 1 : 1 mit den gleichen Blutarten vorbehandelt. Beide Versuche sind in Tabelle 8 zusammengestellt.

TABELLE 8.

Haemagglutination von Meerschweinchen- und Hühnerblut durch Media von Stamm 6, 65 und 108 nach Adsorption der Media durch verschiedene Blutarten.

Adsorption mit:		Medium Coli 6	Medium Coli 65	Medium Coli 65	Medium Coli 108	Medium Coli 65	Medium Coli 65
		Agglutination von Meerschweinchenblut				Agglutination von Hühnerblut	
Meerschweinchen-Blut	3 : 1		>1/8			>1/8	
	1 : 1	>1/4		>1/4	>1/4		>1/4
	1 : 2	1/8		1/128	>1/4		1/32
	1 : 10	1/64		1/128	1/4		1/128
Hühner-Blut	3 : 1		>1/8			>1/8	
	1 : 1	1/64		1/64	>1/4		>1/4
	1 : 2	1/128		1/128	>1/4		1/16
	1 : 10	1/64		1/256	1/4		1/128
Menschen-Blut	3 : 1		1/256			>1/8	
	1 : 1	1/64		1/512	>1/4		1/64
	1 : 2	1/128		1/256	>1/4		1/64
	1 : 10	1/64		1/512	1/4		1/128
Hammel-Blut	3 : 1		1/256			1/128	
	1 : 1	1/64		1/64	1/64		1/64
	1 : 2	1/32		1/128	1/32		1/64
	1 : 10	1/128		1/256			1/128
Nicht adsorbierte Controlle		1/64	1/256	1/256	1/32	1/128	1/128

Hierbei zeigte sich ein deutlicher Unterschied zwischen Coli 6 und Coli 108. Während das Medium von Stamm 6 durch Vorbehandlung mit Hühner-, Menschen- und Hammelblut unbeeinflusst blieb und nur durch Vorbehandlung mit Meerschweinchenblut in den Verhältnissen 1 : 1 und 1 : 2 seine Haemagglutinine verlor, wurden aus dem Medium von Stamm 108 auch durch Hühner- und Menschenblut die Haemagglutinine entfernt. Nur Hammelblut war ohne Einfluss.

Besonders interessant waren die Ergebnisse bei dem Medium von Coli 65. Die Agglutinine gegenüber Meerschweinchenblut wurden durch einmalige und dreimalige Adsorption mit Meerschweinchenblut 1 : 1 völlig entfernt, aber schon eine Adsorption im Verhältnis 1 : 2 war unwirksam. Ferner wurden die Haemagglutinine gegenüber Meerschweinchenblut durch Adsorption mit Hühnerblut nur sehr wenig vermindert, erst eine dreimalige Adsorption entfernte diese völlig. Mit Menschen- und Hammelblut liess sich kein Agglutinin aus dem Medium entfernen. Anders war es mit dem Agglutinin gegenüber Hühnerblut. Adsorption im Verhältnis 1 : 1 mit Meerschweinchen- und Hühnerblut entfernte alle Agglutinine, Adsorption im Verhältnis 1 : 2 zeigte eine geringe Wirkung. Menschenblut dagegen entfernte nach dreimaliger Adsorption alle Hühner-Haemagglutinine, während es auf die Meerschweinchen-Haemagglutinine ohne Wirkung geblieben war. Nur Hammelblut erwies sich als wirkungslos gegenüber den Hühnerblut-Haemagglutininen.

Zusammenfassung.

Von 105 Stämmen von *E. coli*, die auf Haemagglutinine gegen Blut von Meerschweinchen, Huhn, Mensch und Hammel untersucht wurden, agglutinierten 53 Stämme mindestens eine der Blutarten in der Endconcentration von 1/16. 10 Stämme agglutinierten alle vier Blutarten. Bei 3 Stämmen gingen die Haemagglutinine in das Medium über. Die Bacterien dieser Gruppe adsorbierten nie die Haemagglutinine aus den Medien, trotzdem die drei Stämme serologisch nicht identisch waren. Diese Erscheinung wurde als gruppenspecifische Nicht-Adsorption bezeichnet. Nach Denaturierung der Bacterien kam es zur Adsorption. Durch Adsorption mit Blut von Meerschweinchen, Huhn und Mensch liessen sich die Haemagglutinine mehr oder weniger aus dem Medium entfernen, nicht aber durch Hammelblut.

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INVESTIGATION INTO THE COMBINED ACTION OF PENICILLIN AND STREPTOMYCIN IN VITRO

by

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In the present investigation laboratory and clinical aspects of the combined action of penicillin and streptomycin are compared. As to the effectiveness of this combination in the treatment of *Str. faecalis endocarditis*, there is a striking unanimity. JAWETZ and GUNNISON (1952) in U.S.A., GARROD (1953) in Britain, CHABBERT (1953) in France suppose this effectiveness to be due to a large increase in early bactericidal action. Laboratory evidence showed that concentrations of penicillin and streptomycin failing to sterilise when acting alone, are effective when acting together.

To ascertain the ratio in which organisms are killed when treated with penicillin, streptomycin or both, a sample must be transferred on a medium free from antibiotics. We used the replica method of LEDERBERG and LEDERBERG (1952) recently introduced for this purpose by ELEK and HILSON (1954).

In the course of this investigation we got evidence that the relative ineffectiveness of penicillin is due to a small number of organisms surviving the action of this antibiotic. Some experiments regarding the character of these "survivors" are included in this investigation.

Clinical references are produced to confirm our laboratory results.

MATERIALS AND METHODS.

Bacterial strains. Professor GARROD kindly sent us three strains of *Str. faecalis* (Bl, Ke and Mo) isolated from endocarditis patients (CATES, CHRISTIE and GARROD, 1951). *Str. viridans* 935 and 275 were isolated in this laboratory from the blood of endocarditis patients. A blood culture of a sepsis patient in the Leiden University Hospital yielded *Staph. aureus* 1375.

The strains 275 and 935 were considered to be *Str. viridans*, because they did not ferment sorbitol, glycerol, mannitol. They did ferment however esculin. Moreover these strains did not resist heating to 60°C., they did not grow at 45°C., nor on the tellurite medium, nor in broth containing 6.5% NaCl, nor in broth pH 9.6. They were sensitive to 0.2 and 0.3 U/ml of penicillin. We could confirm the physiologic characteristics of the three strains of *Str. faecalis* as described by CATES, CHRISTIE and GARROD (1951). For two of these strains (Bl and Mo) the minimum inhibitory concentration of penicillin was in our hands slightly higher (5 U/ml). Lancefields' group D serum was not available to us.

Media. Nutrient agar 3%, 5% sheep blood agar, heated blood agar (Levinthal agar).

Inoculation. The strains were inoculated in a few ml of broth on top of a 3% agar column. By this means we aimed at obtaining constant growth conditions (pH, food supply, waste removal; DINGER, 1934). Early next morning the plates were inoculated with this culture, one drop per 10 ml of medium being spread on the surface. We call these plates primary plates.

Antibiotics. We received fresh penicillin solution every week through the courtesy of the N.V. Koninklijke Nederlandse Gist en Spiritus Fabriek, Delft. Originally we used streptomycin made by "Specia", in more recent experiments streptomycin from the Delft factory. In a special experiment with regard to their effectiveness no difference was found between both streptomycins.

Sensitivity tests were performed according to the diffusion method. Discs 6 mm in diameter containing 1/100 ml of one antibiotic or 2/100 ml of a mixture of two were placed on the plate after one hour and removed after another hour. The sensitivity discs were freshly prepared for each experiment. They contained 5 U of penicillin (for *Str. faecalis* 100 U) and 25 μg of streptomycin (for *Str. faecalis* 500 μg) or a combination of these quantities.

Replicating. Organisms are transferred by a stamp from the surface of the primary plate to a medium free from antibiotics (replica plate). Colonies appearing after overnight incubating on the replica plate originate from colonies, microcolonies or single bacteria on the primary plate. Unlike LEDERBERG and LEDERBERG, and ELEK and HILSON (who replicated with velvet) we used ordinary white blotting paper, glued on a brass stamp 40 mm in diameter.

Paper. White blotting paper R 19 (Rikkers) 0.5 mm in thickness. Discs cut 6 mm in diameter for sensitivity discs, 40 mm for replicating. The paper is sterilised at 100°C. for 2 hours. After use the paper is destroyed.

EXPERIMENTAL.

I. Testing the amount of penicillin transferred by the paper disc. If from an uninoculated medium containing penicillin in high concentrations a replica is made on another medium free from antibiotic, penicillin-sensitive organisms are neither killed nor inhibited on this replica plate. So no significant amount of antibiotic is transferred by the paper disc.

II. Ratio of organisms replicated. Two sets of plates A and B are inoculated alike. Be B the primary plates, that are replicated consecutively on C and D using the same paper disc. The ratio in which the organisms are transferred is deduced from the number of colonies found after overnight incubating on C or D and the number found on the control plates (A). This ratio is found to be between 1 in 8, and 1 in 10 for the first replica (C) and between 1 and 40, and 1 in 90 for the second one (D). These results are better than those with velvet as recorded by Elek and Hilson who transferred an average of 1 in 40 on the first replica plate.

TABLE 1.

Numbers of colonies after 18 hours' incubation of *Staph. aureus* 1375 in various dilutions.

dilution of culture	10^{-4}	$\frac{10^{-4}}{2}$	10^{-5}
control plates (A)	600	250	90
primary plate (B)	500	230	68
1st replica (C)	65	30	10
2nd replica (D)	15	3	1

III. Replica sensitivity tests. The primary plates are inoculated in the morning; after one hour the sensitivity discs are placed on the plates and left for one hour. Approximately 7 hrs after the first contact between the antibiotic(s) and the organisms, the first replica is made. After overnight incubation a ring of continuous growth is seen on the replica plate. The diameter of the inhibition zone on the primary plate as well as the inner diameter of the ring of continuous growth on the replica plate are then measured. The ring on the replica plate may be larger or smaller than the inhibition zone. If the colonies that can be seen at the boundary of the zone of inhibition have been killed by the expanding antibiotic, the diameter of the ring on the replica plate will be larger than the one of the zone of inhibition. Conversely it will be smaller if the replica is made before the boundary of the zone of inhibition is established, or if within this zone numerous organisms are suppressed but not killed by bacteriostatic concentrations of the antibiotic.

Fig. 1 and 2 give an impression of a replica made 7 hours after the first contact of antibiotic(s) and organisms. The replica plate was incubated overnight at 37°C. Generally the staphylococci were cultured and replicated on nutrient agar, *Str. viridans* and *Str. faecalis* on blood agar and Levinthal's agar. Small differences between *Staph. aureus* and *Str. faecalis* will be discussed later on (p. 325). The following observations bear on penicillin-sensitive *Staph. aureus*, *Str. viridans* and *Str. faecalis*, as results obtained with these strains agreed very closely.

Replica after 7 hours.

1) penicillin: the inner diameter of the ring of continuous growth is large, yet inside the ring numerous organisms are not killed.
2) streptomycin: the zone inside the ring is smaller but practically free from survivors.
3) chlortetracycline, chloramphenicol, oxytetracycline: the inner diameter of the ring is much smaller than the zone of inhibition on the original plate; there is a large number of surviving bacteria inside the ring.
4) penicillin and streptomycin: the ring has the same inner diameter as with penicillin, within the ring survivors are rare.
5) penicillin and chlortetracycline or chloramphenicol or oxytetracycline: inner diameter of the ring just as large as with penicillin, inside the ring numerous survivors.

Replicas made 24 hours after the first contact of the antibiotic with the organisms closely resemble those made after a 7 hours' interval. The number of colonies inside the ring decreases. For chlortetracycline, chloramphenicol and oxytetracycline the inner

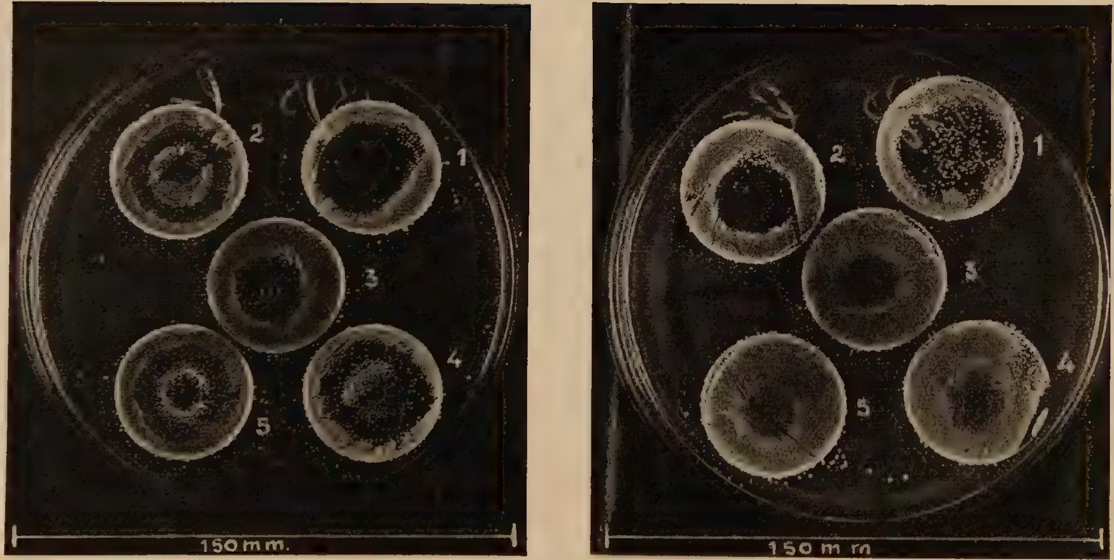

Fig. 1 and 2. *Staph. aureus* 1375. Replica of zone of different antibiotics made after 7 hours incubation, read after another 17 hours.

Fig. 1.

1. penicillin
2. streptomycin
3. oxytetracycline
4. chloramphenicol
5. chlortetracycline

Fig. 2.

1. penicillin + streptomycin
2. penicillin + chlortetracycline
3. streptomycin + chloramphenicol
4. penicillin + oxytetracycline
5. penicillin + chloramphenicol

diameter of the ring increases but does not exceed the diameter of the zone of inhibition.

The results of penicillin in combination with chloramphenicol or one of the tetracyclines being essentially the same we have only recorded in table 2 the data for the penicillin-chloramphenicol combination.

These data indicate that apparently penicillin and streptomycin together kill the organisms more rapidly than penicillin acting alone or in combination with chloramphenicol or the tetracyclines. As the diameter of the zone free from survivors is larger for the combination penicillin and streptomycin than for streptomycin alone, it seems that small ineffective concentrations of streptomycin are bactericidal when acting in cooperation with penicillin.

Even after a 5 × 24 hours contact with the strongly bactericidal combination of penicillin and streptomycin a few colonies of enterococci inside the ring could be noted on the replica plate. Moreover it appears that after a contact of 24—48 hours with a combination of penicillin and chloramphenicol etc., the majority of the organisms is unable to survive. The main difference with the highly bactericidal combination of penicillin and streptomycin seems to lie in the rate of killing. We could confirm these results for other penicillin-sensitive staphylococci and streptococci.

IV. Origin of the colonies inside the ring of continuous growth on the replicaplate.

We are inclined to connect them with the "persisters" recorded by Bigger (1944). Bigger's observation that these organisms are just as sensitive as the original strain was confirmed by our own experiments. The interpretation, however, that these bacteria are accidentally occurring resting bacteria and so do not fall a prey to penicillin, did not satisfy us. Our considerations are:

1. Resting bacteria are not killed by the penicillin-streptomycin combination (Hunter, 1950 and Reynolds and Rowley, 1953). In our own experiments we observed no lethal effect on a culture of *Staph. aureus* 1375 at 4°C.
2. The position of the surviving colonies inside the ring is striking, the colonies being grouped mainly in the centre, but failing nearly completely in the periphery (fig. 3).
3. It is not clear why after a lapse of 24—48 hours the greater part of these survivors is no longer viable.

TABLE 2.

Comparison of diameters on primary plates and replica plates. Assesment of number of colonies.

antibiotic	*Str. viridans* 935 on bloodagar			*Staph. aureus* 1375 on nutrient agar		
	Primary plate (2 observations)	replica plates		primary plate (2 observations)	replica plates	
		7 h.	24 h.		7 h.	24 h.
P	25	26^{++}	37–	28	27^{++}	36±
	25	29^{++}	38–	27	29∾	37–
S	12	12	25–	22	22±±	23–
	11	14–	19	22	21–	25±
A	17	8	20^{+++}	14	14±±	12^{++}
	18	8–	15	13	12^{++}	12∾
C	20	6^{+}	13^{+}	22	15±±	18^{+}
	19	5–	17–	22	8–	16±±
T	20	7–	8	18	12^{++}	12^{+}
	18	7–	14–	15	8^{+}	9^{+}
PS	24	30±	40–	27	28±±	34–
	23	28±	37	28	25–	37–
PC	24	25∾	36^{+}	27	28∾	35^{++}
	25	27∾	36^{+}	28	25∾	38^{++}

antibiotic	*Str. faecalis* on Levinthal agar				
	strain	primary plate	replica plates		
			7 h.	24 h.	5 × 24 h.
P	Bl		19∾	21^{++}	23–
	Mo		32∾	22^{++}	25–
	Ke		21∾	23±±	28
S	Bl	15	12–	13^{+++}	
	Mo	18	15∾	17∾	
	Ke	14	13∾	13∾	
PS	Bl		20–	23^{+}	24–
	Mo		25–	25±	25–
	Ke		24–	25±	26–

1— 10 colonies –
10— 20 colonies at the periphery 1) of the diameter ±
10— 20 colonies in the centre of the diameter +
20— 50 colonies at the periphery 1) ±±
20— 50 colonies in the centre ++
50—100 colonies in the centre +++
>100 ∾

P = penicillin
S = streptomycin
A = chlortetracycline
C = chloramphenicol
T = oxytetracycline
PS = penicillin + streptomycin
PC = penicillin + chloramphenicol

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1) The reason why peripheral colonies may be less important than central will be commented upon further on.

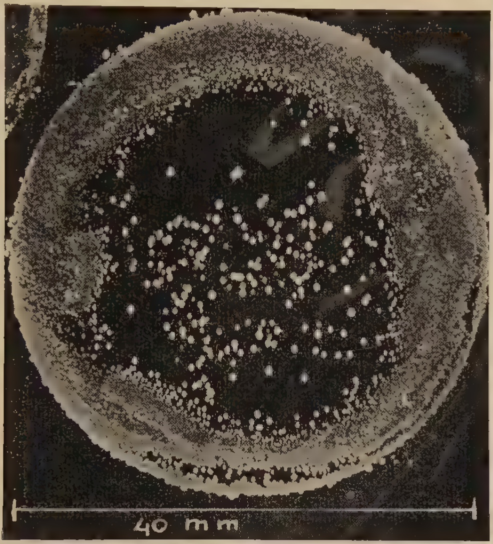

Fig. 3. *Staph. aureus* 1375. Replica of penicillin zone made after 7 hours incubation, read after another 17 hours.

A culture of *Staph. aureus* 1375 was spread on nutrient agar media containing penicillin in increasing concentrations (1/100—10 U per ml). This strain is sensitive to 8/100 U of penicillin, only rare or-

TABLE 3.

Number of colonies of *Staph. aureus* 1375 counted after contact with inincreasing concentrations of penicillin.

Penicillin concentration	replica 6 h.	replica 9 h.	replica 24 h.
1/100	∾	∾	∾
2/100	∾	∾	∾
4/100	++++	+++	+++
8/100	+	80	80
32/100	++	110	15
1	++++	+	35
2	++++	+	100
5	+++	170	40
10	++++	450	100
control	∾	∾	∾

+ = ± 600 colonies
++ = 1000—1500 colonies
+++ = 1500—2000 colonies
++++ = 2000—3000 colonies
∾ = > 3000 colonies

ganisms being resistant to this concentration on the primary plates. After 6, 9 and 24 hours replicas were made (table 3).

The lowest number of viable bacteria is noted at 8/100 U/ml and the numbers increase at higher concentrations, so 8/100 U/ml may be considered optimal. After 24 hours the difference between the numbers of the bacteria surviving the optimal or the higher concentrations is less marked. This experiment was repeated several times with the same result.

The grouping observed on the replica of the penicillin zone may be interpreted in yet another way:

The diffusion method entails naturally that bacteria more remote from the sensitivity-disc divide more frequently than those in the centre before they get into contact with the antibiotic (SCHÖNFELD, 1953). Should there be any connection between the phase of growth and the number of survivors, this number would be far smaller in a culture that has been growing for 2 hours before being brought into contact with penicillin than in organisms mixed with penicillin at the very beginning of their incubation.

To test this, media were inoculated with *Staph. aureus* 1375. On three plates we placed directly, on three others after a lapse of 2 hours, discs of paper (40 mm in diameter) containing penicillin in such concentrations that under the disc 8/100 U/ml or higher concentrations of penicillin were present in the medium. The results are recorded in table 4.

TABLE 4.

Number of survivors of *Staph. areus* 1375 on area (40 mm in diameter) of agar medium to which penicillin is added directly and 2 hours after inoculation.

Penicillin concentration in the disc (U/ml)	0 hrs	2 hrs
1	30	7
5	25	15
20	25	15

The number of survivors replicated from a 2 hours' culture is only slightly smaller than the number found viable when taken from a 0 hours' culture. The replicas were made after 8 hours. The accumulation of colonies in the centre of the ring on the replica plate is in our opinion due to the difference in efficacy between optimal concentrations (in the periphery) and higher concentrations (in the centre of the diffusion zone). This same difference was found for *Str. viridans* 275. With the concentrations of penicillin used, it was not observed for *Str. faecalis*. Accordingly, in a 7 hours' replica of a diffusion test of the *Str. faecalis* strains the organisms surviving the action of penicillin are more evenly distributed inside the ring. In the central area where the sensitivity-disc (6 mm in diameter) had been placed, they were generally lacking.

If a zone of inhibition is replicated twice in succession, the difference between the numbers of the survivors on both plates is negligible. If the organisms are sufficiently dispersed on the primary plate, the second replica is known to transfer only 1 in 4 to 1 in 10 of the number of organisms of the first one. This made it all the more doubtful that such colonies on the replica plate would originate from single resting bacteria.

For further investigation *Staph. aureus* 1375 was spread on two agar media containing 8/100 U/ml of penicillin. After 6 hours' incubation six consecutive replicas were made of one plate (1, 2, 3a, 3b, 4a, 4b), and after 10 hours 8 replicas (1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b) of the second plate. The suffix a and b indicate that two replicas were made with the same paper disc. The replica plates were incubated overnight. The next morning a number of the colonies on the last replica plate was registered on a glass-sheet. By

TABLE 5.

Tracing of colonies originating from survivors on 8 consecutive replica plates. The original plate had been incubated for 10 hours.

Replica	Number of the colony												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1a	1	1	1	1	1	1	1	1		1	1	1	1
1b	1	1	1	1	1	1	1	1	1	1	1	1	1
2a	1	1			1	1		1	1		1	1	
2b	1	1	1	1	1	1	1	1	1		1	1	1
3a	1	1	1		1		1	1	1		1		1
3b	1	1	1	1	1	1	1	1				1	1
4a			1		1		1	1					
4b	1	1	1	1		1			1	1	1		
	7×	7×	7×	5×	7×	6×	6×	7×	5×	3×	6×	5×	5×

this means we could track down the corresponding colonies on the other replica plates. Each colony that has been found on at least two other plates has been taken up in table 5.

On the replica plates made after 6 hours' incubation 6 colonies could be traced 5 times, 5 colonies 4 times, 1 colony 3 times.

The results strongly suggest that these colonies do not originate from resting bacteria (BIGGER's ,,persisters'') but from microcolonies formed in spite of the contact with penicillin.

V. What is the fate of the "persisters" at various concentrations? Table 6 enlightens this subject.

TABLE 6.

The fate of the survivors at various concentrations and after various intervals of time.

	Staph. aureus 1375				*Str. viridans* 275				*Str. faecalis* Ke				*Str. faecalis* Mo			
Penicillin concentration U/ml	0.04	0.08	0.32	2	0.08	0.32	2	4	0.32	2	4	10	2	4	10	20
replica 7 hrs	$\backsim$	850	$\underset{\cdots}{\backsim}$	$\underset{\cdots}{\backsim}$		2200	$\underset{\cdots}{\backsim}$	$\underset{\cdots}{\backsim}$	$\backsim$	$\underset{\cdots}{\backsim}$	2600	240	$\underset{\cdots}{\backsim}$	$\underset{\cdots}{\backsim}$	1250	1300
replica 24 hrs	$\underset{\cdots}{\backsim}$	25	27	14	60	3	15	15	$\backsim$	$\underset{\cdots}{\backsim}$	250	30	$\underset{\cdots}{\backsim}$	1200	70	280
replica 48 hrs	$\underset{\cdots}{\backsim}$	40	16	—	85	—	—	—	$\backsim$	$\underset{\cdots}{\backsim}$	200	8	$\underset{\cdots}{\backsim}$	350	40	80
replica 72 hrs		30	1	—							85	—		110	7	1

$\underset{\cdots}{\backsim}$ plates completely covered, separate colonies visible.

Fewer staphylococci survive an "optimal" concentration than a higher one; 2 U of penicillin kill these survivors within 48 hours. After 72 hours we transferred 30 colonies from a medium containing 8/100 U.

With *Str. viridans* 275 as well as with *Str. faecalis*, Mo and Bl, we observed that higher concentrations of the antibiotic killed the "survivors" more rapidly than the minimal inhibitory (*Str. faecalis*) or the optimal concentration (*Str. viridans*), thus the higher concentrations are more efficient destroyers. For this reason we consider the colonies at the boundary of the diffusion zone (Table 3) of minor importance.

As might be objected that after 48—72 hours' incubation the concentration of penicillin in the medium would have sunk below the inhibitory level, the following experiments were carried out: 1. Of two media free from bacteria containing 8/100 U of penicillin one was placed at 37°C., the other at 4°C., for three days. Thereafter both were inoculated with a 1/1000 dilution of a 24 hours' culture of *Staph. aureus* 1375 and incubated overnight. No growth

was observed on either medium. The conclusion is that the concentration of penicillin has not fallen below the inhibitory level.
2. Broth in a series containing penicillin in gradually increasing concentrations was inoculated with *Staph. aureus* 1375. After 24 hours a sample was transferred to a medium free from antibiotics, the tubes were centrifuged, the supernatant was replaced by an equal concentration of penicillin in fresh broth, and this was repeated twice with an interval of 24 hours (table 7).

TABLE 7.

The fate of the organisms surviving the contact of daily renewed penicillin. Number of organisms transferred on an agar medium and counted after 18 hours incubation.

Penicillin concentration U/ml	4/100	8/100	16/100	32/100	64/100	1	2	5	10
24 hrs.	∞	65	90	125	70	75	65	25	35
48 hrs.	∞	∞[1]	3	—	3	7	9	10	2
72 hrs.	∞	∞	—	—	—	—	—	—	—

The results of these experiments invalidate the objection that the perseverance of the survivors at optimal concentrations is due to a decrease of the concentration of penicillin in the medium.

Discussion.

The penicillin-streptomycin combination is unanimously considered to be the treatment of choice for *Str. faecalis endocarditis*. For *Str. viridans* on the other hand, until recently the clinical authors recommended long-term huge doses penicillin treatment. In Finland's (1954) opinion large doses are indispensable to sustain efficient concentrations in walled-off foci where the organisms grow slowly and are less vulnerable. King *et al.* (1949) failed to cure 7 out of 8 *Str. viridans endocarditis* patients with massive doses of penicillin during 10 days. They did succeed however with moderate doses of penicillin for 5 to 6 weeks, or with penicillin and streptomycin. Geraci and Martin (1953) record good results of a two weeks combined treatment with 2 million U of penicillin and 2 g of dihydro-streptomycin daily in 23 *Str. viridans endocarditis* patients.

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[1] It should be born in mind that the ∞ bacteria may have originated from one single organism resistant to 8/100 U of penicillin.

Weinstein, Daikos and Perrin (1951) found that in fibrin clots the concentration of penicillin was directly related to the size of the intramuscular dose. The increase of concentration was slower in the clot than in the blood, but so was the decrease.

Our conclusion is that the relative inefficacy of the penicillin treatment of subacute endocarditis is due to the existence of ,,survivors" such as we met with in vitro and not to organisms hiding in walled-off foci. The inability of low doses of penicillin to kill the organisms hiding in the vegetations as supposed by Finland, needs experimental confirmation. It seems difficult to explain with this theory, why smaller concentrations of penicillin when combined with streptomycin have a lethal effect on these "snipers".

The possibility that in vivo the "survivors" are longer viable when in contact with penicillin than in vitro, should be considered.

There are strong reasons to identify our "survivors" with Bigger's persisters. We cannot endorse Bigger's opinion that these persisters are resting bacteria. They must have divided in spite of penicillin and have formed microcolonies. Further investigations into the character of these "survivors" are necessary.

Unlike Finland (1954) we consider these organisms of unquestionable importance with regard to the effectiveness of penicillin in vivo. The benefit of a combined penicillin-streptomycin treatment of *Str. viridans endocarditis* is stressed by Hunter (1950), Loewe, Cohen, Eiber (1953), Haynal and Mosonyi (1952), Geraci and Martin (1953). In conformity with Eagle's theory of aggregate time (1949) the results of our experiments suggest that the length of the period during which penicillin is given, is decisive.

It is to be expected that the penicillin-streptomycin combination gives better results than penicillin combined with chloramphenicol or tetracyclines in those diseases in which the drug alone must effect a thorough and swift destruction of the invading bacteria without help of the defensive mechanism of the body.

In his review Finland records only a few successful cures by penicillin in combination with one of the so called bacteriostatic antibiotics. On the other hand, however, he mentions the experience of many others who failed to cure endocarditis patients with these combinations or with the bacteriostatic antibiotics alone, but who succeeded afterwards with penicillin alone or in combination with streptomycin.

Our conclusion that the difference between the action of penicillin and streptomycin on the one hand and penicillin alone or in combination with the bacteriostatic antibiotics is mainly a difference of degree and of time, is very well compatible with these clinical data. The contrast between "synergistic" and "antagonistic" combination (JAWETZ) should not be overemphasized.

We have confirmed the "zone phenomenon" for *Staph. aureus* 1375 (sensitive to penicillin) and for *Str. viridans*, if transferred to a medium free from antibiotics within 12 hours after the first contact of the organisms and the antibiotic. The practical significance of this phenomenon is questionable. On the one hand it is true that higher concentrations lead to more survivors, on the other hand they destroy these survivors more rapidly. Our results are in agreement with those of EAGLE (1948, table 2). At optimal concentrations, the lethal effect as evinced by the number of survivors is superior to that at higher concentrations, when transfer takes place after 6 hours' incubation. From that point onwards it alters gradually in favour of the higher concentrations.

One of FINLAND's conclusions seems worth to be stressed. He found both with penicillin and streptomycin a good correlation between the in vitro sensitivity of the organisms and the prognosis of the patient, but not between the sensitivity and the dosage necessary to obtain a good result. With the so called bacteriostatic antibiotics there was no correlation with either the prognosis or the curative dosage. Once again (SCHÖNFELD, 1953) we may conclude that if the correlation with the clinical results is questionable, the accuracy as pursued in the in vitro sensitivity tests is of merely theoretical importance. More reviews like FINLAND's giving both laboratory details and clinical results are urgently needed.

Summary.

1. With the so called replica method (LEDERBERG and LEDERBERG, 1952) a significant difference was found between the effect on *Str. faecalis*, *Str. viridans* and penicillin-sensitive *Staph. aureus*, of the penicillin-streptomycin combination on the one hand and penicillin alone or in combination with chlortetracycline, chloramphenicol or oxytetracycline on the other. This difference may be connected with the presence of the so called "persisters" (BIGGER, 1944).

2. The theory that these organisms, surviving an 8 hours' contact with penicillin are resting, non-multiplying bacteria escaping

by chance the lethal effect of the antibiotic is incorrect. Evidence is produced that these bacteria have divided in spite of the influence of penicillin.

3. EAGLE's "zone phenomenon" was confirmed for *Staph. aureus* and *Str. viridans* if the contact with the antibiotic did not surpass 12 hours. Thereafter higher concentrations were found to be more effective than "optimal" ones.

4. Publications are quoted which show that the combination penicillin-streptomycin gives better clinical results in the treatment of *Str. viridans endocarditis* patients than penicillin alone which is in accordance with the results of our experiments.

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EXPERIENCES WITH THE TREPONEMAL IMMOBILIZATION TEST IN THE NETHERLANDS

by

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(Received January 13, 1955).

Since the introduction of the treponemal immobilization (TPI) test by NELSON and MAYER in 1949 a great number of communications concerning the value of this test for the diagnosis of syphilis have been published (MAGNUSON and THOMPSON, 1949; MOORE and MOHR, 1952; MILLER *et al.*, 1954; DUREL *et al.*, 1952; REYN and NIELSEN, 1952; CHACKO, 1953; WILKINSON, 1954 and many others).

In this study the results obtained during 1954 in the Laboratory of Bacteriology and Serology of the National Institute of Public Health at Utrecht, are reported.

MATERIALS AND METHODS.

The sera examined came from patients at various stages of syphilis, treated and untreated; from patients suspected of giving non-specific results with the standard serum (STS) tests for syphilis and from patients presumed of being non-syphilitic.

The TPI technique described by NELSON *et al.* (1949, 1951) and by SAUSSE (1951) has been followed with only minor modifications ¹). Improved survival of the treponemes was obtained by increasing five times the amount of sodium thioglycollate (NIELSEN, 1953; PORTNOY *et al.*, 1953) and by replacing the serum ultrafiltrate and the saline by normal rabbit serum. The tests were performed qualitatively and the results expressed in terms of specific immobilization (IS) considering the test to be negative when the IS was

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¹) The Nichols strain employed was obtained through the courtesy of Dr P. DUREL, Hôpital Saint-Lazare, Paris and one of us (B) visited this hospital to study the TPI test.

below 20 per cent, doubtful when it was between 20 and 50 per cent and positive when it was above 50 per cent.

The majority of the sera was tested repeatedly on the same specimen; significant differences in IS were only seldom encountered. Where the TPI tests could not be interpreted (e.g. treponematocidal action of the serum) repeat samples of the patient were tested.

The reagins in the sera were demonstrated simultaneously by the Kolmer complement fixation test and the V.D.R.L. tube flocculation test using cardiolipin antigens prepared in our laboratory.

RESULTS.

A total of 226 sera was examined representing 67 sera from patients with untreated late symptomatic or asymptomatic syphilis; 72 sera from patients with syphilis treated at various stages of the disease and 87 sera from patients with diseases other than syphilis or presumed to be non-syphilitic.

Untreated syphilis.

Table 1 records the results of 67 sera.

TABLE 1.

Comparison of simultaneous TPI tests and STS tests in untreated late syphilis.

Origin of sera	STS	TPI				total
		+	±	—	inconclusive	
Tertiary syphilis	+	6	0	0	1	7
	—	4	0	0	0	4
Neurosyphilis	+	3	0	0	0	3
	—	0	0	0	0	0
Latent syphilis	+	36	2	0	10	48
	—	0	0	0	0	0
Congenital syphilis	+	4	0	0	0	4
	—	0	0	1	0	1
						67

From this table it appears that, excluding unsatisfactory TPI results, of a total of 56 cases of untreated late syphilis 55 were TPI reactive (53 positive and 2 doubtful). The only case with both negative TPI and STS results is as yet obscure. It concerns a girl, aged 12, with an interstitial keratitis, whose mother, however, is TPI and STS negative and showed never any sign of a syphilitic infection.

Treated syphilis.

In table 2 the results of 72 sera are reported.

TABLE 2.
Comparison of simultaneous TPI tests and STS tests in treated syphilis.

Origin of sera	STS	TPI				total
		+	±	—	inconclusive	
Primary syphilis	+	0	0	0	0	0
	—	0	0	5	0	5
Secondary syphilis	+	0	0	0	0	0
	—	0	0	2	0	2
Tertiary syphilis	+	8	0	0	0	8
	—	1	0	0	0	1
Neurosyphilis	+	2	0	0	0	2
	—	1	1	0	0	2
Latent syphilis	+	35	0	3	2	40
	—	6	0	0	1	7
Congenital syphilis	+	5	0	0	0	5
	—	0	0	0	0	0
						72

The 72 patients in this series had all been treated before performance of the TPI test. Of 7 patients with treated early syphilis, either primary or secondary, all were TPI negative. All 9 patients with tertiary syphilis were TPI positive and of 4 patients with neurosyphilis 3 were TPI positive and one doubtful.

Among 47 patients with latent syphilis 41 were TPI positive, 3 were negative and 3 inconclusive. All 5 patients with congenital syphilis were TPI positive.

Non-syphilitic conditions.

In table 3 data on 87 sera from patients with diseases other than syphilis and presumably non-syphilitic individuals are represented.

It appears from this table that, excluding unsatisfactory results, of a total of 79 patients 74 were TPI negative, 14 of which were

TABLE 3.

Comparison of simultaneous TPI tests and STS tests in presumably non-syphilitic conditions.

STS	TPI				total
	+	±	—	inconclusive	
+	1	1	14	4	20
—	0	3	60	4	67
					87

STS positive (biologic false?). The case with both positive TPI and STS tests concerns a donor of a bloodtransfusion service who never showed signs or history of syphilis. The case with the doubtful TPI test and the positive STS tests concerns a pregnant woman without any signs or history of syphilis. Three patients gave doubtful TPI and negative STS results. One was a woman with a neurotic depression; the second was a donor of a bloodtransfusion service and the third was an Indo-European woman.

In the last case the possibility of another treponematosis can not with certainty be excluded.

Discussion.

Although the material presented in this study is small, the results obtained compare favourably with those communicated by other investigators (Zellmann, 1954).

In untreated late syphilis the TPI antibody is uniformly present in the serum regardless of the STS result. Of 56 sera in our material both TPI and STS tests were positive in 49 cases; both negative in one case and TPI positive-STS negative in 4 cases. In 2 cases reagin was present in the serum while the TPI test was doubtful.

In treated early syphilis the TPI antibody and the reagin may disappear from the serum as a result of adequate therapy and in our study this was the case in our 7 patients with primary or secondary syphilis.

In treated late symptomatic and asymptomatic syphilis the TPI antibody is almost invariably present and treatment has little effect on the TPI test.

Among the 60 sera in our material TPI antibody was present in 54 cases. In 3 cases the TPI test was negative while reagin was still present and in the remaining 3 cases the TPI test could not be interpreted (toxicity of the sera). In the 5 cases of congenital syphilitic infection of our study both TPI antibody and reagin were present.

From the literature the conclusion can be drawn that in non-syphilitic persons a positive TPI test is a rarity. In our series of 87 sera one positive and 4 doubtful results were found. Although in these cases a syphilitic infection was very unlikely the possibility of syphilis or of another treponematosis could not completely be excluded. It must be kept in mind that these 87 sera represent selected cases where in other laboratories positive or doubtful STS tests were found without clinical signs of syphilis, which STS results, however, could not always be confirmed in our laboratory.

Conclusions and summary.

A survey is given of the results obtained by the TPI tests carried out during 1954 in the Netherlands.

On the basis of the 226 sera studied simultaneously with TPI test and the STS tests it can be concluded:

1. In untreated late symptomatic or asymptomatic syphilis the TPI test is uniformly positive regardless the STS tests. A negative TPI test in cases of suspected syphilis renders this diagnosis improbable.
2. In treated early syphilis, primary and secondary, the TPI test like the STS tests may become negative after adequate therapy.
3. In treated late symptomatic or asymptomatic syphilis including congenital syphilis the TPI test is almost invariably positive in spite of therapy.
4. As in non-syphilitic conditions a positive TPI test is very rare, the TPI test can be assumed as highly specific and can be used for reliable differentiating between syphilis or another treponematosis and diseases capable of producing biologic false positive STS tests.

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UNTERSUCHUNGEN ÜBER DIE PHYSIOLOGIE DER WEINHEFEN. VI. MITTEILUNG

WEITERE UNTERSUCHUNGEN ÜBER DIE BEDEUTUNG DER STICKSTOFF- BZW. ARGONBEGASUNG UND DES ZUSATZES KLEINER SAUERSTOFFMENGEN FÜR DIE VERGÄRUNG DER GLUCOSE DURCH DIE WEINHEFE „FENDANT" AUS KULTUREN VERSCHIEDENEN ALTERS. VERGLEICH MIT DER UNTERGÄRIGEN BIERHEFE „MÜLLERBRÄU" UND DER BRENNEREIHEFE „TARANTO" [1]

von

T. WIKÉN und **O. RICHARD**

(Eingegangen am 8. Januar 1955).

Kürzlich wurde gezeigt, dass das anaerobe Gärvermögen der Kulturweinhefen ,,Dézaley", ,,Fendant", ,,Herrliberg", ,,Salenegg" und ,,Polymorphus II" vom Alter der Kulturen, aus denen das betreffende Zellmaterial stammt, stark abhängig ist. Die beträchtlichen Schwankungen, denen die anaerobe Gärgeschwindigkeit somit unterworfen ist, wurden für die ersten vier Weinheferassen von WIKÉN und RICHARD (22) volumetrisch in der Apparatur nach VON EULER, MYRBÄCK, NILSSON und ALM (siehe NILSSON (14)) und für die letzte Rasse von FIECHTER (7) mit Hilfe der manometrischen Methode von BARCROFT, HALDANE und WARBURG (siehe DICKENS (3), DIXON (4), UMBREIT, BURRIS und STAUFFER (18)) festgestellt. Die Messung der anaeroben Kohlendioxydentwicklung wurde dabei in sämtlichen Fällen nach Begasung der Hefesuspensionen mit sauerstofffreiem Stickstoff durchgeführt. Später gelang es THORNE (17) nachzuweisen, dass das Alter der Kulturen, aus denen das Zellmaterial für die Gärversuche geerntet wird, auch bei unter- und obergärigen Brauereihefen einen grossen Einfluss auf das anaerobe Gärvermögen ausübt. Die Geschwindigkeit der Kohlendioxydproduktion wurde dabei in Kohlendioxydatmosphäre nach der volumetrischen Methode von Enebo und Sandegren (6) gemessen. Die grosse Abhängigkeit der aeroben Kohlendioxydabgabe vom Alter der betreffenden Kulturen wurde in manometrischen Versuchen mit ruhenden Zellen der Weinheferassen ,,Herrliberg'', ,,Polymorphus II'' und ,,Steinberg'' von Fiechter (7, 8) festgestellt.

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[1] I, II, III, IV, V. Mitteilung: Antonie van Leeuwenhoek **17**, 209, 1951; **18**, 31, 1952; **18**, 293, 1952; **19**, 279, 1953; **20**, 385, 1954.

Ferner konnten Wikén und Richard (23, 24) zeigen, dass folgende Unterschiede bestehen zwischen dem Zymasesystem der aus jungen (17—20 Std. alten) ,,Fendant''-Kulturen geernteten Zellen und demjenigen der Zellen, welche aus älteren (66—72 Std. alten) Kulturen der gleichen Hefe stammen:

1. Das Gärvermögen des aus jungen Kulturen gewonnenen ruhenden Hefematerials ist grösser in Luft bzw. in Stickstoff mit einem geringen Gehalt an Sauerstoff (0,01—1 Volumen-%) als in sauerstofffreiem Stickstoff.

2. Die Geschwindigkeit der Vergärung der Glucose durch ruhende Zellen aus jungen Kulturen ist unter streng anaeroben Bedingungen von der Art der Stickstoffbegasung abhängig, indem sie bei zunehmender Dauer der Stickstoffbehandlung kräftig abnimmt.

3. Die nach Stickstoffbegasung während 30—40 Min. bei ruhenden Zellen aus jungen Kulturen eintretende Gärungshemmung ist reversibel, denn sie lässt sich durch geringe Sauerstoffmengen (0,01—1 Volumen-%) beseitigen.

4. Nach lange dauernder Stickstoffbegasung (40 + 20 bzw. 40 + 40 und 40 + 60 Min.) treten bei ruhenden Zellen aus jungen Kulturen sehr starke Gärungshemmungen ein, welche sich nur zum Teil durch kleine Sauerstoffmengen aufheben lassen. Beim Schütteln von Hefesuspensionen, deren Zellen aus jungen Kulturen stammen, in sauerstofffreiem Stickstoff finden mithin nebst der reversiblen, in Anwesenheit geringer Sauerstoffmengen rückgängigen Inaktivierung bestimmter Zymasekomponenten in gewissem Ausmasse Prozesse statt, die zur irreversiblen Zerstörung von Zellbestandteilen führen, welche für die Gärung (Dissimilation des Zuckers ohne Sauerstoffaufnahme) unerlässlich sind.

5. Bei ruhendem Zellmaterial aus alten Kulturen kann der erwähnte Einfluss der Dauer der Stickstoffbegasung bzw. des Zusatzes geringer Sauerstoffmengen auf die Gärgeschwindigkeit nicht oder nur in geringem Ausmasse festgestellt werden. Die Zymase der aus alten Kulturen gewonnenen Hefe zeigt unter streng anaeroben

Bedingungen praktisch die gleiche Leistungsfähigkeit wie in Luft oder in Gemischen von Stickstoff mit kleinen Mengen Sauerstoff.

Es sei schliesslich erwähnt, dass die hier in aller Kürze beschriebenen Effekte auch nach Begasung der Hefesuspensionen mit sauerstofffreiem Argon nachgewiesen werden konnten. Es sei ferner hervorgehoben, dass die Behandlung der Zellsuspensionen mit Luft, bzw. Stickstoff oder Argon während verschiedener Zeitspannen vor dem Einkippen der Glucoselösung stattfand. Unmittelbar vor dem Beimischen des Zuckers erfolgte auch das Zusetzen der kleinen Luft- bzw. Sauerstoffmengen (WIKÉN und RICHARD (23, 24)).

Bekanntlich unterliegt das Kultursubstrat im Laufe des Wachstums bzw. der Vermehrung der Hefe wesentlichen Veränderungen. Beispielsweise nimmt sein Gehalt an Zucker, Stickstoffverbindungen, Mineralsalzen und Spurenelementen ab, während die Konzentration des Aethylalkohols und Kohlendioxyds sowie anderer Endprodukte des Stoffwechsels zunimmt. In diesem Zusammenhang sei ferner daran erinnert, dass das Substrat C, welches zur Gewinnung des von WIKÉN und RICHARD (23,24) untersuchten Hefematerials diente, Caseinhydrolysat, d.h. ein Gemisch von ca. 20 verschiedenen Aminosäuren (siehe BLOCK und BOLLING (2) S. 303), enthält (WIKÉN und RICHARD (20)). Nun ist es bekannt, dass die Geschwindigkeit, mit welcher die wachsenden und sich vermehrenden Hefezellen die einzelnen Komponenten eines „natürlichen" oder synthetischen Aminosäuregemisches aufnehmen, sehr stark von der Art der betreffenden Säuren sowie von ihren relativen Konzentrationen abhängig ist. Beispielsweise zeigte BARTON-WRIGHT (1), dass bei der Vergärung einer bestimmten Bierwürze Methionin bereits nach 24 Stunden zu 100% assimiliert worden war. Nach 48—96 Stunden war Tryptophan zu 99%, Threonin, Isoleucin, Asparaginsäure, Valin, Leucin, Tyrosin, Arginin und Phenylalanin zu 90—94%, Serin, Glutaminsäure, Lysin und Histidin zu 82—85%, Cystin zu 63% und Prolin zu 14% von der Hefe aufgenommen worden. Man darf somit annehmen, dass die aus jungen Kulturen auf Substrat C geernteten Hefezellen unter wesentlich anderen Bedingungen bezüglich der Aminosäurezusammensetzung des Mediums gewachsen sind als der grösste Teil des Zellmaterials, das aus alten Kulturen gewonnen wurde. Es fragte sich deshalb, ob die Unterschiede in der physiologischen Aktivität, welche zwischen Zellmaterial aus jungen und solchem aus alten Kulturen vorliegen, mit den erwähnten Veränderungen des Substrats zusammenhängen, oder von der jeweiligen Zusammensetzung des Mediums unabhängig und für bestimmte Phasen in der Entwicklung der Hefekultur — für bestimmte Abschnitte der S-förmigen Vermehrungskurve — charakteristisch sind. Um diese Frage zu lösen, setzten wir mit der auxo-autotrophen Kulturweinhefe „Fendant" und der auxo-heterotrophen untergärigen Brauereihefe „Müllerbräu" die Experimente an, deren Ergebnisse in Abb. 1, 2 und 3 sowie Tabelle 1 aufgeführt sind.

TABELLE 1.

Behandlung der Hefesuspension vor dem Zusetzen der Glucoselösung. Siehe Legende der Abb. 2.	Approximativ geradliniger Abschnitt der Gärkurve	
	Zeitspanne Min.	Gärgeschwindigkeit ml Kohlendioxyd pro 300 mg feuchte Hefe und Std.
Versuch Abb. 2 a. Zellen aus 17 Std. alten Kulturen		
1	30—160	15,2
2	60—200	13,0
3	30—160	14,9
4	130—320	8,9
5	70—210	13,1
6	200—450	6,6
7	120—260	11,2
8	240—600	5,1
9	140—320	9,6
Versuch Abb. 2 d. Zellen aus 67 Std. alten Kulturen		
1	60—220	12,1
2	100—230	11,5
3	90—210	12,4
4	100—240	11,1
5	100—230	11,5
6	100—240	11,1
7	100—230	11,5
8	100—260	10,9
9	100—240	11,1

Die 17 Std. alten Kulturen wurden mit frischer Nährlösung und die 67 Std. alten mit dem aus diesen jungen Kulturen geernteten Substrat angesetzt (vgl. Legende der Abb. 2).

Aus den Ergebnissen der Versuche über „künstliche Symbiosen", welche mit *Mucor ramannianus* und *Rhodotorula rubra* (Müller und SCHOPFER (12), MÜLLER (11)), *Nematospora gossypii* und *Polyporus adustus* bzw. *Polyporus abietinus* (KÖGL und FRIES (10)) sowie anderen Pilzen, Hefen und Bakterien (siehe SCHOPFER (15) S. 26 und 245 ff). ausgeführt wurden, geht hervor, dass auxo-heterotrophe Mikroorganismen in Mischkulturen auf Substraten ohne irgend einen Zusatz von Wuchsstoffen wachsen und sich vermehren können, indem sie ihren Bedarf an solchen Stoffen gegenseitig decken. Die Zellen von *Mucor ramannianus* sind heterotroph in bezug auf den Thiazol-Teil des Aneurins, synthetisieren aber den Pyrimidin-Teil, während die Zellen von *Rhodotorula rubra* wohl den Thiazol-Teil zu bilden vermögen aber eine Zufuhr des Pyrimidin-Teils von aussen benötigen. Die Tatsache, dass in Mischkulturen, welche mit wuchsstofffreien Substraten angelegt werden, Wachstum beider Organismen stattfindet, zeigt, dass die Pilz- bzw. Hefezellen zur Ausscheidung der betreffenden Aneurin-Komponenten fähig sind. Diese liegen sodann in aktiver Form im Substrat vor und können von der Hefe bzw. vom Pilz aufgenommen werden. In der gleichen Weise liefert *Nematospora gossypii* (Biotin-heterotroph) Aneurin und die *Polyporus*-Arten (Aneurin-heterotroph) Biotin, wodurch das Wachstum in Mischkultur in synthetischer Nährlösung ohne Wuchsstoffzusatz ermöglicht wird. Im Hinblick auf diese Befunde schien es nun von Interesse zu prüfen, ob die von uns beim Zellmaterial aus jungen Weinhefekulturen festgestellte Empfindlichkeit für Begasung mit sauerstofffreiem Stickstoff bzw. Argon auf die Abwesenheit einer spezifischen Substanz zurückzuführen ist, welche in den aus alten Kulturen stammenden unempfindlichen Zellen vorliegt und aus deren intakten Strukturen freigemacht und durch die zytoplasmatische Schicht sowie die Zellwand in das Medium ausgeschieden werden kann. In Gemischen von Zellen aus jungen und solchen aus alten Kulturen sollten die ersten, welche den spezifischen Stoff nicht oder höchstens in minimalen Mengen enthalten, diesen aus dem Medium aufnehmen können und sodann die gleichen Gäreigenschaften aufweisen wie die aus alten Kulturen gewonnenen Zellen. Vorausgesetzt dass diese eine für beide Zellarten hinreichende Menge der betreffenden Substanz enthalten, sollte das Zellgemisch gegen die Begasung mit sauerstofffreiem Stickstoff bzw. Argon unempfindlich sein. Zur Prüfung dieser Hypothese wurden mit der Kulturweinhefe ,,Fendant'' die Versuche ausgeführt, deren Ergebnisse in Abb. 4, 5 und 6 sowie Tabelle 2 zusammengestellt sind.

WIKÉN und ZAHNER (25) haben die Empfindlichkeit des aus

TABELLE 2.

	Zusammensetzung der Hefesuspension %		Volumen Kohlendioxyd entwickelt während der Zeitspanne 120—240 Min.			
	Zellmaterial aus alten Kulturen	Zellmaterial aus jungen Kulturen	Gefunden		Berechnet	
Versuch Abb. 4			In Luft	In Stickstoff	In Luft	In Stickstoff
	100	0	21,7	21,6	—	—
	95	5	21,8	20,0	21,1	20,8
	70	30	17,8	15,6	18,3	16,8
	30	70	13,4	9,5	13,9	10,3
	5	95	10,9	5,8	11,1	6,3
	0	100	10,5	5,5	—	—
Versuch Abb. 5			In Luft	In Argon	In Luft	In Argon
	100	0	20,0	21,6	—	—
	95	5	20,5	20,3	20,0	21,3
	70	30	21,2	18,7	20,0	19,9
	30	70	22,2	17,4	20,0	17,6
	5	95	20,8	16,3	20,0	16,2
	0	100	20,1	15,9	—	—
Versuch Abb. 6			In Argon			
	100	0	21,0		—	
	95	5	20,4		20,4	
	70	30	17,3		17,7	
	30	70	11,7		13,0	
	5	95	10,3		10,8	
	0	100	10,3		—	

Die Bebrütungszeit der Kulturen und die Behandlung der Hefesuspensionen vor dem Zusetzen der Zuckerlösung gehen aus der Legende der Abb. 4, 5 und 6 hervor.

jungen Kulturen geernteten Zellmaterials für Stickstoff- bzw. Argonbehandlung bei weiteren sporulierenden Weinhefen, und zwar der auxo-heterotrophen Rasse ,,Dézaley'' und der auxo-autotrophen Rasse ,,Salenegg'', feststellen können. Die Zellen, welche aus alten Kulturen dieser Hefen stammten, waren gleich wie das entsprechende Zellmaterial der Rasse ,,Fendant'' (Wikén und Richard (23, 24)) unempfindlich. In der vorliegenden Arbeit wurde nebst der

Weinhefe ,,Fendant'' und der Brauereihefe ,,Müllerbräu ''eine weitere Rasse, und zwar die Brennereihefe ,,Taranto'', preliminär geprüft. Die Ergebnisse liegen in Abb. 7 vor.

METHODIK.

Bezüglich der zur Gewinnung des Zellmaterials und zur Durchführung der Gärversuche verwendeten Methoden verweisen wir auf die Mitteilungen I, III, IV und V in dieser Zeitschrift (20, 21, 22, 23). Für die Kulturen der auxo-autotrophen Weinhefe ,,Fendant'' kam die Nährlösung C ohne irgend einen Zusatz von Wuchsstoffen zur Verwendung, während im Falle der untergärigen Brauereihefe ,,Müllerbräu'' und der Brennereihefe ,,Taranto'', welche sich als auxo-heterotrophe Rassen erwiesen, das Substrat mit einem Gemisch der Vitamine (+)-Biotin, meso-Inosit und D(+)-Pantothensäure (25 γ bzw. 25 mg und 2,5 mg/1000 ml) versetzt wurde.

Die Anordnung der Versuche über das Gärvermögen des jungen und alten Zellmaterials, das in frischer Nährlösung bzw. Substrat aus jungen und alten Kulturen gezüchtet worden war, geht aus der Legende der Abb. 1, 2 und 3 hervor. Hier sei nur noch erwähnt, dass die aus jungen bzw. alten Kulturen geernteten Substrate durch einen Jena-Glasfiltertiegel G 4 filtriert wurden. Die ersten Substrate kamen sodann direkt nach dem Dämpfen als Medien für neue Kulturen zur Verwendung, während die letzten Substrate vor dem Sterilisieren mit Glucose und im Falle der auxo-heterotrophen Rasse ,,Müllerbräu'' auch mit Wuchsstoffen ergänzt wurden. Ferner wurde das pH auf 4,8—5,0 eingestellt.

Quantitative Analysen mit Hilfe der Halbmikromethode von STILES, PETERSON und FRED (16) ergaben für die aus alten Hefekulturen geernteten Substrate einen Glucosegehalt von 4—6 g pro 1000 ml. Das frische Substrat C enthält 50 g Glucose pro 1000 ml (WIKÉN und RICHARD (20)). Die erwähnten Substrate wurden demnach mit 44—46 g Zucker pro 1000 ml versetzt.

Hier sei nur noch hinzugefügt, dass die Geschwindigkeit der Kohlendioxydproduktion der 2mal mit destilliertem Wasser und 1mal mit Bernsteinsäure-Natriumsuccinat-Puffer (pH 4,9) gewaschenen Hefezellen volumetrisch in der Apparatur nach VON EULER, MYRBÄCK, NILSSON und ALM bei 25°C. gemessen wurde (NILSSON (14); siehe WIKÉN und RICHARD (22, 23)). Dabei enthielten die Gärgefässe in sämtlichen Experimenten 300 mg frische Hefe und 200 mg wasserfreie Glucose. In den Abbildungen 1—7 sind als Mass des Gärvermögens die abgelesenen Kohlendioxydvolumina ohne Umrechnung auf Normalbedingungen angegeben. Diese Umrechnung erübrigt sich, da nur die Geschwindigkeiten gleichzeitig durchgeführter und somit unter identischen Druck- und Temperaturbedingungen verlaufender Gärungen miteinander verglichen werden.

Die Bestimmung des Trockensubstanzgehalts der Hefe erfolgte gemäss der Methode von Brandt (siehe Wikén und Richard (22)).

ERGEBNISSE UND DISKUSSION.

Versuche mit jungem und altem Zellmaterial aus Hefekulturen in frischer Nährlösung sowie im Substrat aus jungen und alten Kulturen.

In der ersten Versuchsreihe, deren Ergebnisse aus Abb. 1 hervorgehen, wurde die „Fendant"-Hefe geprüft. Es kam Zellmaterial aus 20 und 68 Std. alten Kulturen auf frischem Substrat (Abb. 1a und b) sowie 16 und 66 Std. bzw. 21 und 66 Std. altes Material, das in den aus den ersten (Abb. 1c und d) bzw. den letzten Kulturen (Abb. 1e und f) geernteten Substraten gezüchtet worden war, zur Verwendung.

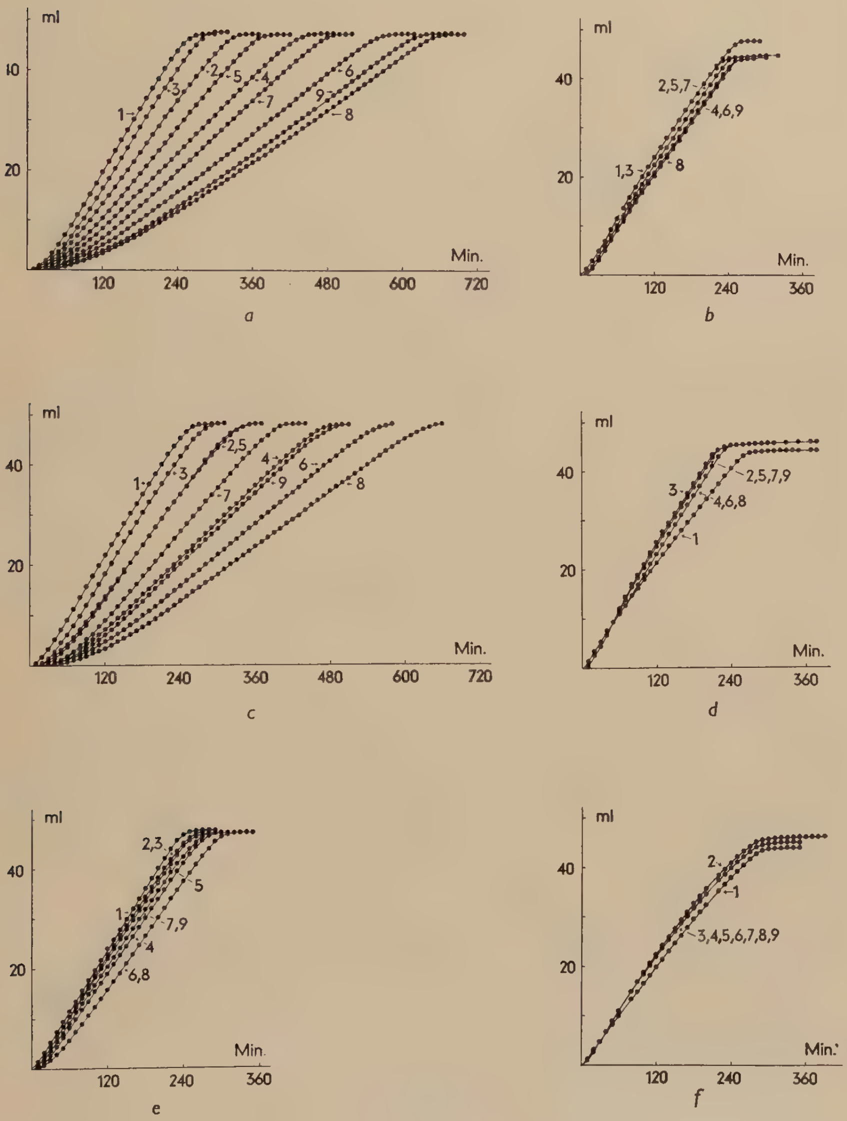
Die Resultate dieser Versuchsreihe zeigen eindeutig, dass die Zellen der „Fendant"-Hefe aus jungen Kulturen sehr empfindlich gegen die Begasung mit sauerstofffreiem Stickstoff sind, gleichgültig

Abb. 1. Die Vergärung der Glucose in Luft (1) bzw. Stickstoff (2, 4, 6 und 8) und Luft-Stickstoff-Gemisch (3, 5, 7 und 9) durch die Weinheferasse „Fendant". Die Abhängigkeit der Gärgeschwindigkeit von der Dauer der N_2-Begasung und vom Zusatz einer kleinen Menge Luft unmittelbar vor dem Einkippen der Zuckerlösung.

a. Zellmaterial aus 20 Std. alten Kulturen auf frischem Substrat.
b. Zellmaterial aus 68 Std. alten Kulturen auf frischem Substrat.
c. Zellmaterial aus 16 Std. alten Kulturen auf dem Substrat aus den 20 Std. alten Kulturen.
d. Zellmaterial aus 66 Std. alten Kulturen auf dem Substrat aus den 20 Std. alten Kulturen.
e. Zellmaterial aus 21 Std. alten Kulturen auf dem ergänzten Substrat aus den 68 Std. alten Kulturen.
f. Zellmaterial aus 66 Std. alten Kulturen auf dem ergänzten Substrat aus den 68 Std. alten Kulturen.

Behandlung der Hefesuspension vor dem Zusetzen der Glucoselösung:

1. 40 Min. Schütteln in ruhender Luft.
2. 40 Min. Schütteln in strömendem Stickstoff.
3. 40 Min. Schütteln in strömendem Stickstoff, Zusatz von 3 ml Luft.
4. 40 Min. Schütteln in strömendem Stickstoff, 20 Min. Schütteln in ruhender N_2-Atmosphäre.
5. 40 Min. Schütteln in strömendem Stickstoff, 20 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.
6. 40 Min. Schütteln in strömendem Stickstoff, 40 Min. Schütteln in ruhender N_2-Atmosphäre.
7. 40 Min. Schütteln in strömendem Stickstoff, 40 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.
8. 40 Min. Schütteln in strömendem Stickstoff, 60 Min. Schütteln in ruhender N_2-Atmosphäre.
9. 40 Min. Schütteln in strömendem Stickstoff, 60 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.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f

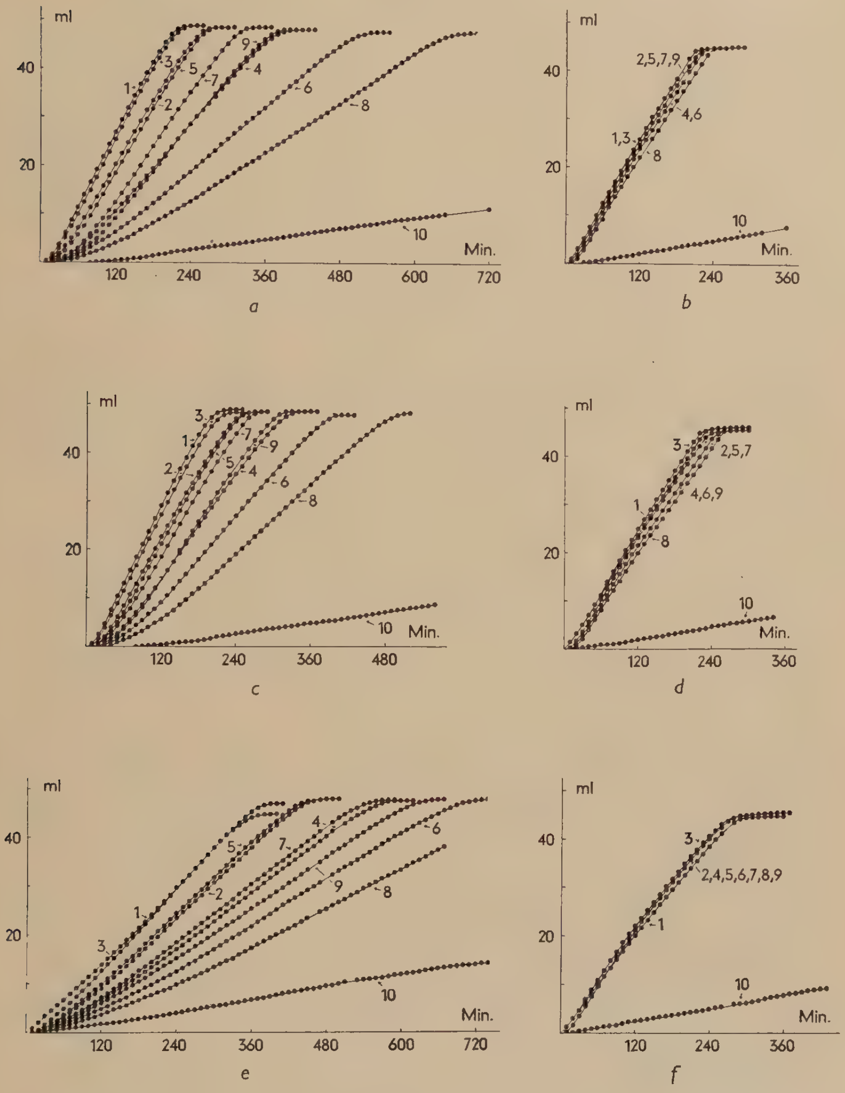
Abb. 1.

ob diese Kulturen mit frischer Nährlösung oder Substrat aus jungen oder alten Kulturen angesetzt werden. Es ist ferner ersichtlich, dass die durch Stickstoffbegasung hervorgerufene Hemmung der Gärgeschwindigkeit nach Zusatz einer kleinen Menge Luft (entsprechend ca. 1 Volumen-% O_2 in der Gasphase der Gärkolben) ganz oder zum Teil aufgehoben wird (Abb. 1a, c und e). Beim Zellmaterial aus alten Kulturen treten diese Erscheinungen nicht oder nur in geringem Ausmasse auf (Abb. 1b, d und f). Während die Inaktivierung bzw. Aktivierung des Zymasesystems des jungen Zellmaterials in den Versuchen mit Hefe aus frischer Nährlösung bzw. dem aus jungen Kulturen geernteten Substrat ausserordentlich stark zum Vorschein kommen (Abb. 1a und c), treten diese Effekte im Experiment mit dem jungen Hefematerial, das im Substrat aus den alten Kulturen gewachsen war, zwar ganz deutlich aber doch in geringerem Umfange auf (Abb. 1e). Um abzuklären, ob dies auf eine Abhängigkeit der Gäreigenschaften des jungen Hefematerials von der Zusammensetzung des Kultursubstrats zurückzuführen ist, oder damit zusammenhängt, dass die Hefe ein wenig zu spät geerntet wurde, setzten wir eine zweite Versuchsreihe mit der Rasse ,,Fendant'' an. Aus den Ergebnissen, welche in Abb. 2 aufgeführt sind, ist ersichtlich, dass es sich nicht um eine Abhängigkeit des

Abb. 2. Die Vergärung der Glucose in Luft (1) bzw. Stickstoff (2, 4, 6 und 8) und Luft-Stickstoff-Gemisch (3, 5, 7 und 9) durch die Weinheferasse ,,Fendant''. Die Abhängigkeit der Gärgeschwindigkeit von der Dauer der N_2-Begasung und vom Zusatz einer kleinen Menge Luft unmittelbar vor dem Einkippen der Zuckerlösung. Kurven 1—9: CO_2-Produktion. Kurve 10: O_2-Aufnahme.

a. Zellmaterial aus 17 Std. alten Kulturen auf frischem Substrat.
b. Zellmaterial aus 66 Std. alten Kulturen auf frischem Substrat.
c. Zellmaterial aus 18 Std. alten Kulturen auf dem Substrat aus den 17 Std. alten Kulturen.
d. Zellmaterial aus 67 Std. alten Kulturen auf dem Substrat aus den 17 Std. alten Kulturen.
e. Zellmaterial aus 18 Std. alten Kulturen auf dem ergänzten Substrat aus den 66 Std. alten Kulturen.
f. Zellmaterial aus 67 Std. alten Kulturen auf dem ergänzten Substrat aus den 66 Std. alten Kulturen.

Behandlung der Hefesuspension vor dem Zusetzen der Glucoselösung:
1. 40 Min. Schütteln in ruhender Luft.
2. 40 Min. Schütteln in strömendem Stickstoff.
3. 40 Min. Schütteln in strömendem Stickstoff, Zusatz von 3 ml Luft.
4. 40 Min. Schütteln in strömendem Stickstoff, 20 Min. Schütteln in ruhender N_2-Atmosphäre.
5. 40 Min. Schütteln in strömendem Stickstoff, 20 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.
6. 40 Min. Schütteln in strömendem Stickstoff, 40 Min. Schütteln in ruhender N_2-Atmosphäre.
7. 40 Min. Schütteln in strömendem Stickstoff, 40 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.
8. 40 Min. Schütteln in strömendem Stickstoff, 60 Min. Schütteln in ruhender N_2-Atmosphäre.
9. 40 Min. Schütteln in strömendem Stickstoff, 60 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.
10. 40 Min. Schütteln in ruhender Luft.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f

Abb. 2.

Gärvermögens vom Kulturmedium handelt. Die mit zunehmender Dauer der Stickstoffbegasung gesteigerte Hemmung der Gärgeschwindigkeit sowie die durch geringe Sauerstoffmengen hervorgerufene Stimulierung dieser Geschwindigkeit sind bei dem jungen, im Substrat aus alten Kulturen gezüchteten Zellmaterial (Abb. 2e) von der gleichen Grössenordnung wie beim jungen Material, das in frischer Nährlösung bzw. dem Substrat aus jungen Kulturen (Abb. 2a bzw. c) gewonnen worden war. Im übrigen werden die Ergebnisse der ersten Versuchsreihe durchwegs bestätigt. Die in Tabelle 1 angeführten Werte, welche aus den in Abb. 2a und d dargestellten Kurven für die Geschwindigkeit der Kohlendioxydproduktion in der annähernd linearen Gärphase berechnet wurden, geben Aufschluss über die Grösse der hier beschriebenen Effekte.

Die Ergebnisse einer mit der auxo-heterotrophen untergärigen Brauereihefe ,,Müllerbräu'' durchgeführten Versuchsreihe liegen in Abb. 3 vor. Es ist ersichtlich, dass sich diese Hefe bezüglich der aeroben und anaeroben Gärfähigkeit des aus jungen und alten Kulturen stammenden Zellmaterials grundsätzlich gleich wie die auxo-autotrophe Kulturweinhefe ,,Fendant'' verhält. Die Gärungshemmungen, welche nach lange dauernder Stickstoffbegasung beim jungen Zellmaterial der ,,Müllerbräu''-Hefe festgestellt werden konnten, sind jedoch wesentlich kleiner als die entsprechenden Effekte

Abb. 3. Die Vergärung der Glucose in Luft (1) bzw. Stickstoff (2, 4, 6 und 8) und Luft-Stickstoff-Gemisch (3, 5, 7 und 9) durch die Bierheferasse ,,Müllerbräu''. Die Abhängigkeit der Gärgeschwindigkeit von der Dauer der N_2-Begasung und vom Zusatz einer kleinen Menge Luft unmittelbar vor dem Einkippen der Zuckerlösung. Kurven 1—9: CO_2-Produktion. Kurve 10: O_2-Aufnahme.

a. Zellmaterial aus 15 Std. alten Kulturen auf frischem Substrat.
b. Zellmaterial aus 63 Std. alten Kulturen auf frischem Substrat.
c. Zellmaterial aus 15 Std. alten Kulturen auf dem Substrat aus den 15 Std. alten Kulturen.
d. Zellmaterial aus 65 Std. alten Kulturen auf dem Substrat aus den 15 Std. alten Kulturen.
e. Zellmaterial aus 17 Std. alten Kulturen auf dem ergänzten Substrat aus den 63 Std. alten Kulturen.
f. Zellmaterial aus 65 Std. alten Kulturen auf dem ergänzten Substrat aus den 63 Std. alten Kulturen.

Behandlung der Hefesuspension vor dem Zusetzen der Glucoselösung:

1. 40 Min. Schütteln in ruhender Luft.
2. 40 Min. Schütteln in strömendem Stickstoff.
3. 40 Min. Schütteln in strömendem Stickstoff, Zusatz von 3 ml Luft.
4. 40 Min. Schütteln in strömendem Stickstoff, 20 Min. Schütteln in ruhender N_2-Atmosphäre.
5. 40 Min. Schütteln in strömendem Stickstoff, 20 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.
6. 40 Min. Schütteln in strömendem Stickstoff 40 Min. Schütteln in ruhender N_2-Atmosphäre.
7. 40 Min. Schütteln in strömendem Stickstoff, 40 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.
8. 40 Min. Schütteln in strömendem Stickstoff, 60 Min. Schütteln in ruhender N_2-Atmosphäre.
9. 40 Min. Schütteln in strömendem Stickstoff, 60 Min. Schütteln in ruhender N_2-Atmosphäre, Zusatz von 3 ml Luft.
10. 40 Min. Schütteln in ruhender Luft.

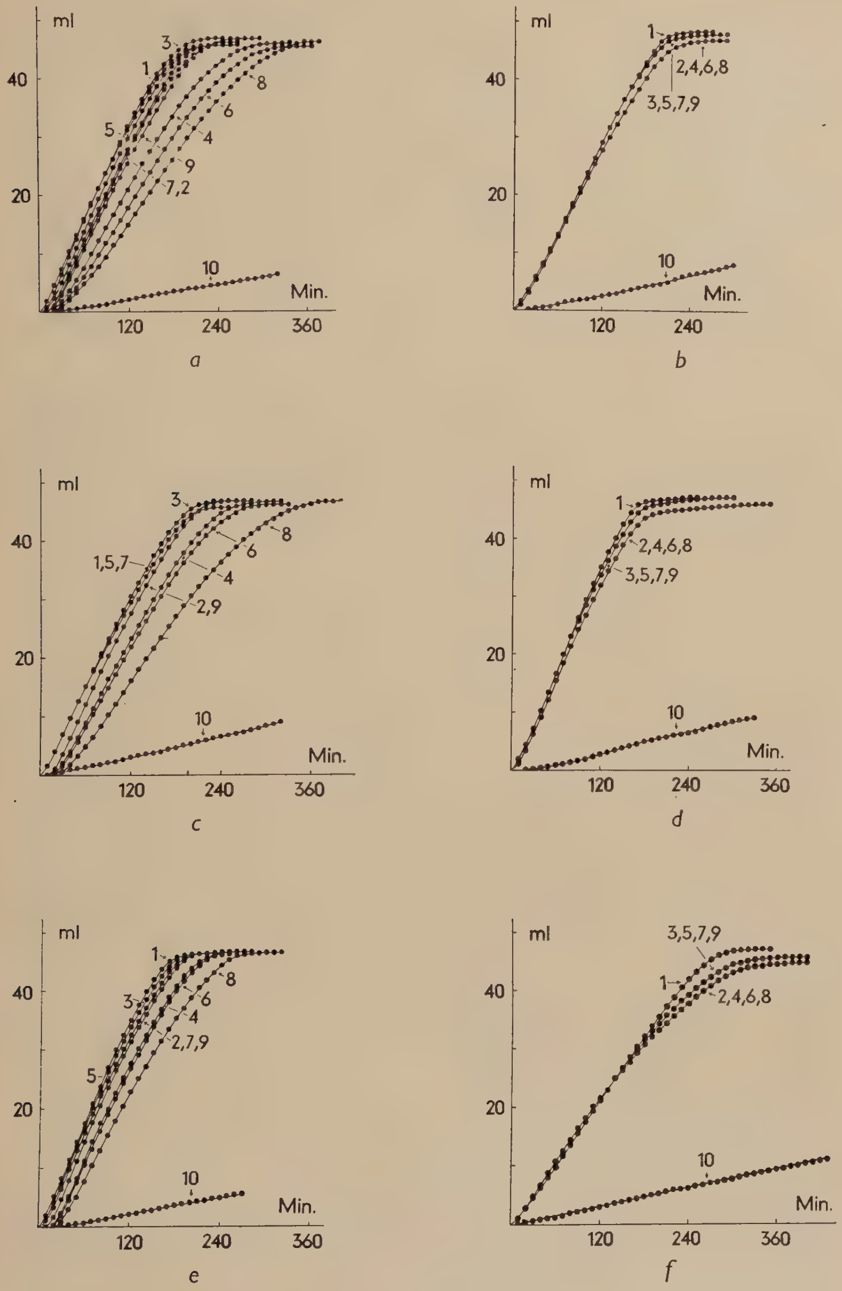
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Abb. 3.

bei der Rasse „Fendant". Ob dies auf eine grössere Widerstandsfähigkeit der Bierhefe gegen Stickstoffbegasung oder auf ein allzu spätes Ernten des geprüften Hefematerials zurückzuführen ist, lässt sich erst nach weiteren Experimenten entscheiden.

Die von uns in früheren Arbeiten (Wikén und Richard (23, 24)) und in der vorliegenden Mitteilung beschriebenen Gäreigenschaften der Heferassen „Fendant" und „Müllerbräu" treten mithin unabhängig von den im Laufe des Wachstums bzw. der Vermehrung der Hefe stattfindenden Veränderungen in der Zusammensetzung des Kultursubstrats auf. Sie sind vielmehr an bestimmte Phasen in der Entwicklung der Hefekulturen — an bestimmte Abschnitte der Wachstums- bzw. Vermehrungskurven — gebunden. In diesem Zusammenhang sei daran erinnert, dass die Zellen zahlreicher Bakterien je nach dem Alter der Kulturen, d.h. je nach der Wachstums- bzw. Vermehrungsphase, in welcher sich die Kulturen befinden, beträchtlichen Schwankungen bezüglich der Grösse und Form sowie der physiologischen Aktivität unterworfen sind (siehe beispielsweise die Uebersichten von Dubos (5) S. 137 ff. und Gunsalus (9) S. 114 ff.). Hier sei ferner erwähnt, dass die beim Zellmaterial aus jungen Kulturen der „Fendant"- und „Müllerbräu"-Hefen festgestellte Aktivierung oder Stimulierung der Gärfähigkeit durch minimale Sauerstoffmengen im Hinblick auf die Diskussion über das Hefewachstum als einen obligat aeroben Prozess von gewissem Interesse ist. Bekanntlich war bereits Beijerinck der Ansicht, dass die Pasteursche Theorie der physiologischen Aequivalenz der Prozesse Gärung und Atmung (Dissimilation des Zuckers ohne bzw. mit O_2-Aufnahme) als nicht ganz einwandfrei zu betrachten ist. In einem am „Nederlandsch Natuur- en Geneeskundig Congres" im Jahre 1887 gehaltenen Vortrag hat Beijerinck die Unerlässlichkeit geringer Mengen des molekularen Sauerstoffs für die Aufrechterhaltung der Lebensfähigkeit aller Organismen hervorgehoben. Nach Beijerinck sind minimale Mengen dieses Gases sogar für einen normalen Verlauf des Wachstums bzw. der Vermehrung der als obligate Anaerobier bezeichneten Bakterien über grössere Zeitspannen unentbehrlich (siehe van Iterson, den Dooren de Jong und Kluyver (19) S. 109 ff.). Gemäss den Untersuchungen von Windisch, Haehn und Heumann (26, 27) sind die als fakultativ anaerob spezifizierten Hefen nicht imstande, sich auf die Dauer ohne die Mitwirkung von molekularem Sauerstoff fortzupflanzen. Die in der vorliegenden Arbeit mitgeteilten Versuchsergebnisse über die Gäreigenschaften des Zellmaterials aus jungen Kulturen der Heferassen „Fendant" und „Müllerbräu" deuten darauf hin, dass die erwähnte Bedeutung des Sauerstoffs wenigstens zum Teil auf seinen aktivierenden oder stimulierenden Einfluss auf eine oder mehrere Komponenten des Zymasesystems in den sich in der Anpassungsphase befindenden Zellen zurückzuführen ist (Anpassungsphase = „period of cellular adjustment" = „initial stationary phase" + „phase of accelerating growth and multiplication rate").

Versuche mit Gemischen von Zellen aus jungen und alten Kulturen der „Fendant"-Hefe.

In diesen Versuchen, deren Ergebnisse in Abb. 4, 5 und 6 sowie Tabelle 2 zusammengestellt sind, wurden die aeroben (Luft) und anaeroben (Stickstoff oder Argon) Gärgeschwindigkeiten von Hefesuspensionen, welche ausschliesslich altes bzw. junges Zellmaterial enthielten, mit denjenigen von Gemischen, die zu 95, 70, 30 und 5% aus dem ersten bzw. zu 5, 30, 70 und 95% aus dem letzten Zellmaterial bestanden, verglichen.

Im ersten Versuch wurden die anaeroben Gärungen in sauerstofffreiem Stickstoff durchgeführt. Wie aus Abb. 4 ersichtlich ist, wurden für die Gemische aus altem und jungem Zellmaterial sowohl unter aeroben als auch unter anaeroben Bedingungen Gärkurven erhalten, welche zwischen denjenigen für die ausschliesslich aus dem ersten bzw. dem letzten Hefematerial bestehenden Suspensionen liegen. Ferner geht aus den in Tabelle 2 aufgeführten experimentell gefundenen bzw. berechneten Werten hervor, dass die Verschiebung der Gärkurven der quantitativen Zusammensetzung der Zellgemische approximativ entspricht.

Im zweiten und dritten Versuch kam anstatt des Stickstoffs sauerstofffreies Argon zur Verwendung. Abb. 5 und 6 sowie Tabelle 2 zeigen, dass die mit Zellgemischen angesetzten Gärungen einen intermediären Verlauf aufweisen. Ihre Geschwindigkeiten lassen sich annähernd additiv aus denjenigen berechnen, welche für das Zellmaterial aus alten bzw. jungen Kulturen festgestellt wurden. Die Uebereinstimmung zwischen den berechneten und experimentell gefundenen Werten kann tatsächlich als befriedigend bezeichnet werden.

Unsere Versuchsergebnisse zeigen mithin eindeutig, dass die aus jungen bzw. alten „Fendant"-Kulturen stammenden Zellen in Ge-

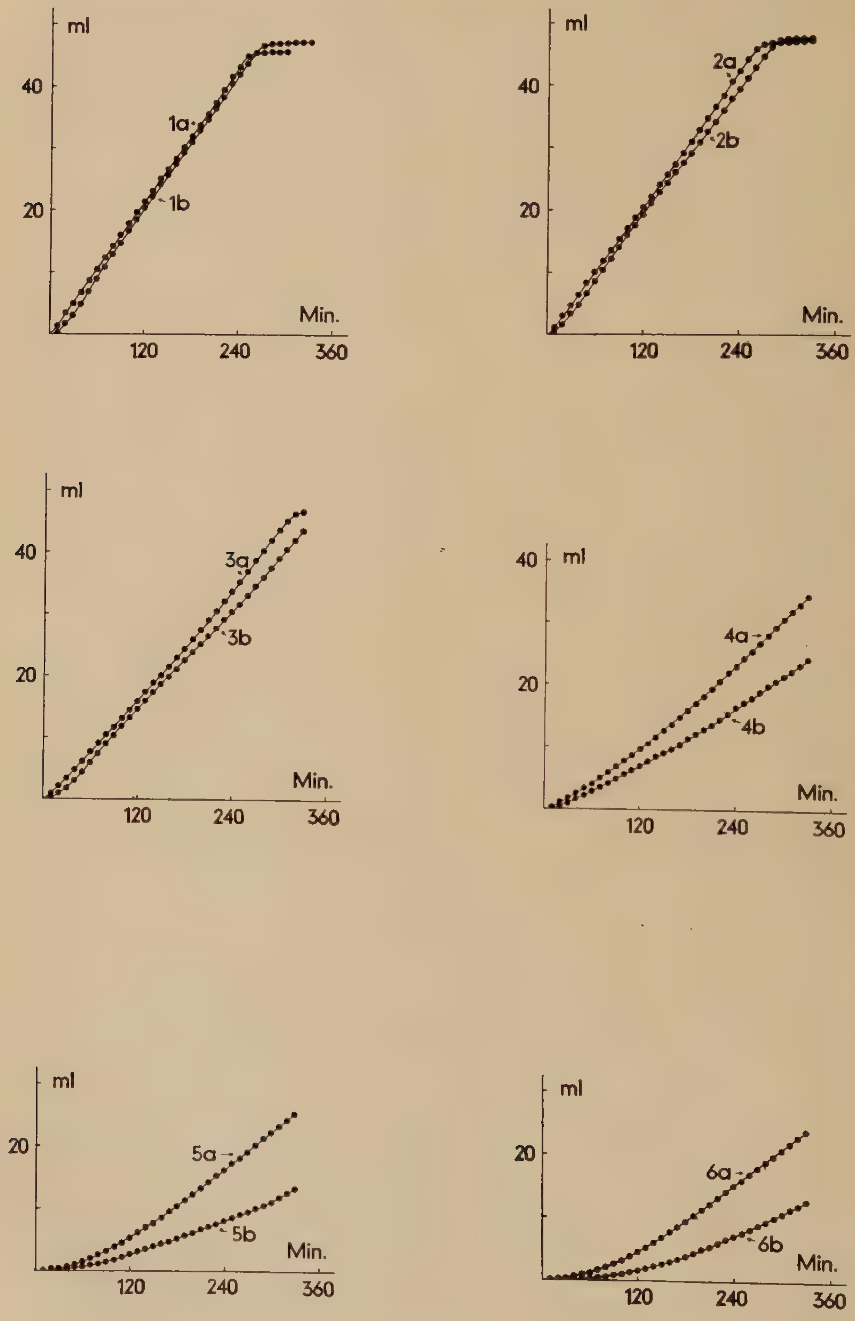
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Abb. 4.

mischen ihre individuellen Gäreigenschaften beibehalten. Die Zellen des alten Hefematerials sind nicht imstande, eine oder mehrere Substanzen auszuscheiden, welche von den aus den jungen Kulturen geernteten Zellen aufgenommen werden und zur Aufhebung ihrer beträchtlichen Empfindlichkeit gegenüber Begasung mit sauerstofffreiem Stickstoff bzw. Argon sowie dem Zusatz geringer Sauerstoffmengen dienen können. Die Komponente oder Komponenten der Zymase und anderer für die Aktivität dieses Enzymkomplexes unerlässlichen Systeme, welche die Unempfindlichkeit des alten Hefematerials bedingen, lassen sich unter den von uns gewählten experimentellen Bedingungen von den intakten Zellstrukturen nicht trennen.

Bekanntlich tritt Acetaldehyd als ein Zwischenprodukt der alkoholischen Gärung der Hefe auf. Er wird ferner als ein Mittel zur Aufhebung der Gärungsinduktion verwendet, und zwar in einer Menge von ca. 0,05 ml 2% Lösung pro 2,0 ml Gärmischung (NILSSON (13, 14)). Die Annahme liegt nahe, dass das von uns geprüfte Zellmaterial aus alten „Fendant"- bzw. „Müllerbräu"-Kulturen verhältnismässig reich an Acetaldehyd ist, während das aus jungen Kulturen gewonnene Material so geringe Aldehydmengen enthält, dass diese beim Waschen der Zellen und durch die darauf folgende Begasung mit strömendem Stickstoff oder Argon je nach der Dauer der letzten Behandlung zum grossen Teil oder sogar restlos entfernt werden. Dabei würden die betreffenden Zellen eine verminderte Gärgeschwindigkeit aufweisen, bis sie aufs neue eine genügende Menge Acetaldehyd gebildet haben. Diese an und für sich nicht unwahrscheinliche Hypothese wird durch unsere Versuchsergebnisse widerlegt (siehe auch WIKÉN und RICHARD (23, 24)). Erstens wird durch

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Abb. 4. Die Vergärung der Glucose in Luft (a) bzw. Stickstoff (b) durch Zellmaterial aus alten (1) bzw. jungen (6) Kulturen der Weinheferasse „Fendant" sowie durch deren Gemische (2, 3, 4 und 5).

Bebrütungszeit der alten Kulturen: 66 Std.
Bebrütungszeit der jungen Kulturen: 17 Std.

Behandlung der Hefesuspensionen vor dem Zusetzen der Glucoselösung:
a. 40 Min. Schütteln in ruhender Luft.
b. 40 Min. Schütteln in strömendem Stickstoff.
1. 100% Zellmaterial aus alten Kulturen.
2. 95% Zellmaterial aus alten Kulturen + 5% Zellmaterial aus jungen Kulturen.
3. 70% Zellmaterial aus alten Kulturen + 30% Zellmaterial aus jungen Kulturen.
4. 30% Zellmaterial aus alten Kulturen + 70% Zellmaterial aus jungen Kulturen.
5. 5% Zellmaterial aus alten Kulturen + 95% Zellmaterial aus jungen Kulturen.
6. 100% Zellmaterial aus jungen Kulturen.

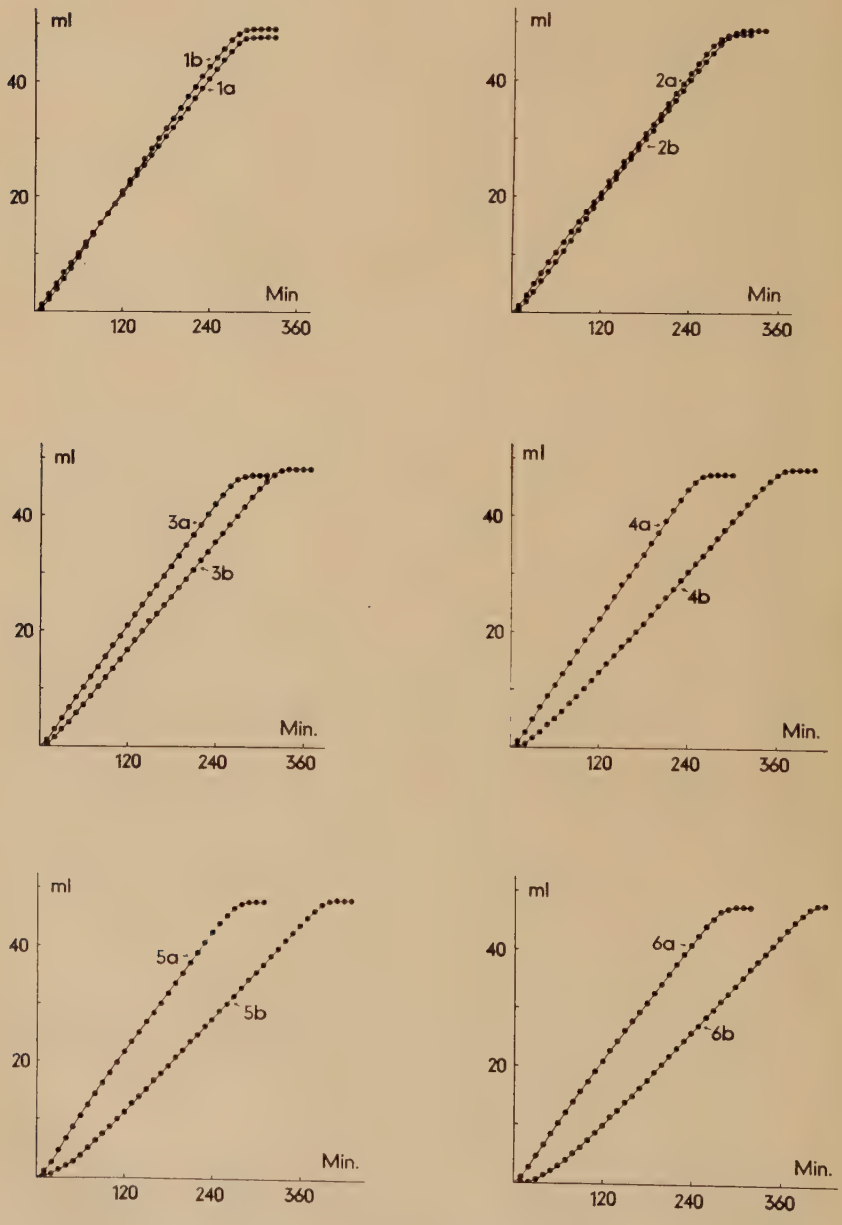
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Abb. 5.

die Stickstoff- bzw. Argonbegasung nicht nur die Induktionsperiode verlängert, sondern auch die Geschwindigkeit der Kohlendioxydproduktion in der approximativ linearen Gärphase herabgesetzt, zweitens werden keine Hemmungseffekte durch Begasung mit strömender Luft hervorgerufen (Wikén und Richard (23) S. 394) und drittens gehört Acetaldehyd zu den Stoffen, welche vom alten Zellmaterial in das Medium ausgeschieden und vom jungen Material aus diesem aufgenommen werden können. Das Gärvermögen des letzten Materials wird aber, wie bereits erwähnt, durch die in den Zellgemischen vorhandenen Zellen aus alten Kulturen nicht stimuliert.

Schliesslich möchten wir an Hand einiger Daten aus dem in Abb. 1 dargelegten Versuch diskutieren, ob die durch Stickstoff- und Argonbegasung bzw. durch Zusatz minimaler Sauerstoffmengen bewirkte Hemmung bzw. Stimulierung des Gärvermögens beim Zellmaterial aus jungen Hefekulturen darauf zurückzuführen ist, dass dieses Material grösstenteils aus den Zellen des Impfmaterials und nur zum geringen Teil aus Zellen besteht, welche im betreffenden Substrat durch Sprossung der Impfzellen entstanden sind. Im erwähnten Versuch kam als Impfmaterial eine Hefesuspension zur Verwendung, die 1,8 (1,83 ± 0,02, 4 Parallelen) mg Hefetrockensubstanz bzw. 7,9 (7,92 ± 0,18, 3 Parallelen) mg trockengeschleuderte frische Hefe pro ml enthielt. Das Substrat jedes Kulturkolbens wurde mit 1 ml dieser Suspension geimpft. Die in frischer Nährlösung angesetzten jungen Kulturen enthielten beim Ernten (nach 20stündiger Bebrütung; Abb. 1a) 83,2 (± 1,0, 4 Parallelen) mg Hefetrockensubstanz bzw. 405,4 (± 3,4, 3 Parallelen) mg trockengeschleuderte frische Hefe pro Kolben. Der Zuwachs in 20 Std. betrug mithin 81,4 bzw. 397,5 mg, berechnet als Trockensubstanz

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Abb. 5. Die Vergärung der Glucose in Luft (a) bzw. Argon (b) durch Zellmaterial aus alten (1) bzw. jungen (6) Kulturen der Weinheferasse „Fendant" sowie durch deren Gemische (2, 3, 4 und 5).

Bebrütungszeit der alten Kulturen: 68 Std.
Bebrütungszeit der jungen Kulturen: 22 Std.

Behandlung der Hefesuspensionen vor dem Zusetzen der Glucoselösung:
a. 40 Min. Schütteln in ruhender Luft.
b. 40 Min. Schütteln in strömendem Argon.

1. 100% Zellmaterial aus alten Kulturen.
2. 95% Zellmaterial aus alten Kulturen + 5% Zellmaterial aus jungen Kulturen.
3. 70% Zellmaterial aus alten Kulturen + 30% Zellmaterial aus jungen Kulturen.
4. 30% Zellmaterial aus alten Kulturen + 70% Zellmaterial aus jungen Kulturen.
5. 5% Zellmaterial aus alten Kulturen + 95% Zellmaterial aus jungen Kulturen.
6. 100% Zellmaterial aus jungen Kulturen.

bzw. frische Hefe. Aus den Werten für das Trocken- bzw. Frischgewicht lässt sich demnach eine 45 bzw. 50 fache Hefevermehrung ermitteln. Dies bedeutet, dass die Impfmenge nur 2% des geernteten Hefematerials ausmacht. Das auf anaerobes bzw. aerobes Gärvermögen geprüfte Material aus jungen Kulturen besteht somit hauptsächlich aus neugebildeten Zellen. Die nach Begasung mit Stickstoff und Argon auftretenden Gärungshemmungen sind gleich wie die durch geringe Sauerstoffmengen hervorgerufene Gärungsstimulierung als Erscheinungen zu betrachten, welche für die in der Anpassungsphase der Hefekulturen entstandenen Zellen charakteristisch sind. Hier sei nur noch erwähnt, dass für die alten (68stündigen) Kulturen in frischer Nährlösung durch quantitative Bestimmung der Hefetrockensubstanz bzw. der trockengeschleuderten frischen Hefe eine 784 bzw. 782fache Vermehrung der Impfzellen festgestellt werden konnte.

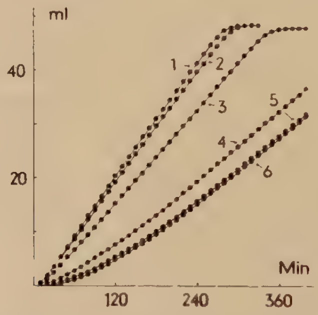

Abb. 6. Die Vergärung der Glucose in Argon durch Zellmaterial aus alten (1) bzw. jungen (6) Kulturen der Weinheferasse „Fendant" sowie durch deren Gemische (2, 3, 4 und 5).

Bebrütungszeit der alten Kulturen: 68 Std.
Bebrütungszeit der jungen Kulturen: 22 Std.
Behandlung der Hefesuspensionen vor dem Zusetzen der Glucoselösung: 40 Min. Schütteln in stromendem Argon.

1. 100% Zellmaterial aus alten Kulturen'
2. 95% Zellmaterial aus alten Kulturen + 5% Zellmaterial aus jungen Kulturen.
3. 70% Zellmaterial aus alten Kulturen + 30% Zellmaterial aus jungen Kulturen.
4. 30% Zellmaterial aus alten Kulturen + 70% Zellmaterial aus jungen Kulturen.
5. 5% Zellmaterial aus alten Kulturen + 95% Zellmaterial aus jungen Kulturen.
6. 100% Zellmaterial aus jungen Kulturen.

Versuche mit Zellen aus jungen und alten Kulturen der „Taranto"-Hefe.

Die Empfindlichkeit des aus jungen Kulturen gewonnenen Zellmaterials für Begasung mit sauerstofffreiem Stickstoff bzw. Argon konnte bis jetzt bei den Weinheferassen „Fendant" (Wikén und Richard (23, 24, diese Arbeit)), „Dézaley" und „Salenegg" (Wi-

KÉN und ZAHNER (25)) sowie bei der Bierhefe „Müllerbräu" (diese Arbeit) festgestellt werden. Wie bereits erwähnt, wurde in der vorliegenden Arbeit ferner die Brennereihefe „Taranto" untersucht, wobei Zellmaterial aus 18 bzw. 70 Std. alten Kulturen in der mit einem Gemisch von (+)-Biotin, meso-Inosit und D(+)-Pantothensäure versetzten Nährlösung C zur Verwendung kam. Aus Abb. 7 geht hervor, dass das geprüfte Material dieser Hefe nach Stickstoffbegasung während 40 Min. keine Gärungshemmung aufweist. Die Brennereihefe „Taranto" ist somit unempfindlich oder durch eine nur kurz dauernde empfindliche Phase im Lebenszyklus der Kulturen gekennzeichnet.

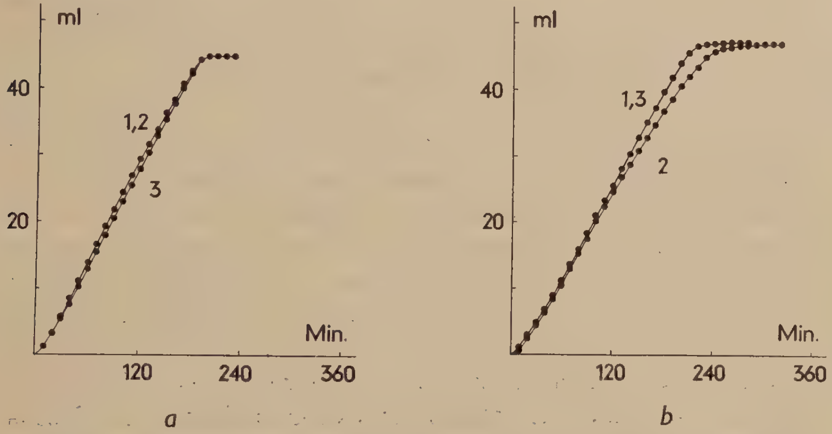

Abb. 7. Die Vergärung der Glucose in Luft (1) bzw. Stickstoff (2) und Luft-Stickstoff-Gemisch (3) durch Zellmaterial aus jungen (a) bzw. alten (b) Kulturen der Brennereihefe „Taranto".

a Zellmaterial aus 18 Std. alten Kulturen.
b. Zellmaterial aus 70 Std. alten Kulturen.

Behandlung der Hefesuspensionen vor dem Zusetzen der Glucoselösung:
1. 40 Min. Schütteln in ruhender Luft.
2. 40 Min. Schütteln in strömendem Stickstoff.
3. 40 Min. Schütteln in strömendem Stickstoff, Zusatz von 3 ml Luft.

Diese Arbeit gehört zum Teil in den Rahmen der Untersuchungen über die Physiologie der Weinhefen, welche von der Abteilung für Landwirtschaft des Eidg. Volkswirtschaftsdepartements aus dem Weinbaufonds unterstützt werden. Den zuständigen Behörden sind wir zu grossem Dank verpflichtet.

Die „Fendant"- und „Taranto"-Hefen erhielten wir von der Eidg. Versuchsanstalt für Obst-, Wein- und Gartenbau, Wädenswil. Die „Müllerbräu"-Hefe wurde uns von der Brauerei Müller AG., Baden, zur Verfügung gestellt. Den Herren Professor Dr F. KOBEL, Dr H. LÜTHI und Dr H. MÜLLER möchten wir für ihre Liebenswürdigkeit herzlich danken.

Fräulein R. BUCHMANN und Fräulein A. MOHLER danken wir bestens für sorgfältige Assistenz.

Summary.

The present paper describes the effects brought about by air, oxygen-free nitrogen and argon as well as mixtures of the two latter gases with small amounts of oxygen (air) upon the fermentation of glucose by resting cells from young (15—22 hours) and old (63—70 hours) cultures of the auxo-autotrophic tame wine yeast Fendant, the auxo-heterotrophic brewery bottom yeast Müllerbräu and the auxo-heterotrophic distillery yeast Taranto.

The general growth and fermentation conditions employed in this investigation are the same as those reported previously (20, 21, 22, 23, 24). However, the Fendant and Müllerbräu yeasts were grown not only in a fresh synthetic nutrient solution containing glucose, vitamin-free casein hydrolysate, potassium citrate buffer, mineral salts and (Müllerbräu, Taranto) a mixture of (+)-biotin, meso-inositol and D(+)-pantothenic acid, but also in substrates harvested from young and old yeast cultures set up with the nutrient solution mentioned. Before use, these substrates were filtered through a sintered glass filter crucible (Jena G 4) and sterilized in flowing steam. Prior to steaming, the substrates obtained from old cultures were supplied with glucose and (Müllerbräu) the above combination of vitamins in order to make up the sugar and growth factor deficiency due to the growth of yeast.

The fermentation capacity of cells from young and old cultures grown in fresh nutrient solution was compared to that of cells harvested from the corresponding cultures on substrates obtained from young and old cultures. In Figures 1—3 and Table 1 the notations are as follows:

a: cells from young cultures b: cells from old cultures	grown in fresh nutrient solution
c: cells from young cultures d: cells from old cultures	grown in substrate from young cultures
e: cells from young cultures f: cells from old cultures	grown in substrate from old cultures

In this way it was possible to decide whether the differences in fermentation capacity existing under anaerobic conditions between cells of young and old cultures (*cf.* below) were due to the changes in amino acid composition of the medium taking place during growth of yeast (1) or to an inherent variation in the physiological proper-

ties of the yeast cells similar to that observed during the growth cycle of bacterial cultures (5, 9).

Further, it seemed interesting to test if the above differences were due to one or more substances which were present in the yeast cells harvested from old cultures and might be liberated from the intact structures of these cells, given off into the medium and taken up by the cells obtained from young cultures of the same yeast. The existence of this phenomenon similar to the exchange of growth factors between auxo-heterotrophic organisms in artificial symbiosis (11, 12, 15) was examined by comparing the aerobic and anaerobic fermentation capacity of yeast suspensions containing mixtures of cells from young and old cultures to that of suspensions prepared with only one type of cells (Figures 4—6, Table 2).

The results of the above experiments may be summarized as follows (*cf.* (23, 24)):

Resting cells from young cultures of the Fendant and Müllerbräu yeasts show a faster fermentation in air and in nitrogen or argon containing small amounts of oxygen than in oxygen-free nitrogen or argon.

Under strongly anaerobic conditions effected by flushing the fermentation flasks with oxygen-free nitrogen or argon before the addition of glucose to the cell suspensions, the rate of fermentation of the cells from young Fendant and Müllerbräu cultures decreases considerably with increasing flushing times. The inactivation of the zymase system brought about by flushing the yeast suspensions with the above gases only for a short period (40 minutes) is completely reversible on addition of small amounts of oxygen, whereas the inhibition of fermentation capacity arising after flushing for longer periods (40 + 20, 40 + 40 and 40 + 60 minutes) is only partly removed on addition of the same gas.

The cells from young cultures of the auxo-heterotrophic brewery bottom yeast Müllerbräu seem to be less sensitive to the inhibiting action of oxygen-free nitrogen than the corresponding cells of the auxo-autotrophic tame wine yeast Fendant. Similar inhibiting effects upon the fermentation of glucose were also observed in experiments with the tame wine yeasts Dézaley and Salenegg (25), the former being an auxo-heterotrophic and the latter an auxo-autotrophic strain (21). On the other hand the cells from young cultures of the auxo-heterotrophic distillery yeast Taranto examined in the present work show the same rate of fermentation in oxygen-free nitrogen as in air. This does not exclude the possibility that cells of this yeast during quite early phases of growth are susceptible to oxygen-free nitrogen or argon.

In experiments with resting cells from old cultures of the Fendant, Dézaley, Salenegg, Müllerbräu and Taranto yeasts no or only very small inhibiting and stimulating effects of the type described above were observed.

The results of the experiments carried out with cells from young and old cultures grown in a fresh medium and in substrates harvested from young and old cultures provide convincing evidence that the sensitivity to flushing with oxygen-free nitrogen or argon and to the addition of minute amounts of oxygen is an inherent physiological property characteristic of yeast cells of the generations developing during quite early phases of the growth cycle. This property is a constituent part of the orderly modification which the yeast population undergoes with age of the culture irrespective of the changes in the amino acid composition of the medium taking place during growth of the yeast. Thus yeast cultures seem to exhibit a "period of physiological youth" or an "embryonic phase" resembling that postulated by several authors for bacterial cultures (5, 9). The stimulating effect produced by minute quantities of oxygen upon the activity of the zymase system of young yeast material flushed with oxygen-free nitrogen or argon seems interesting in view of PASTEUR's hypothesis of the physiological equivalence of fermentation and respiration on the one hand, and BEIJERINCK's hypothesis of the indispensability of small quantities of oxygen for maintenance of life even of facultatively and obligately anaerobic organisms over long periods (19) on the other hand.

The results of the experiments with yeast suspensions containing mixtures of cells from young (5, 30, 70 and 95%) and old cultures (95, 70, 30 and 5%) along with suspensions prepared with only one type of yeast cells show conclusively that the sensitivity of the former and insensitivity of the latter cells to flushing with oxygen-free nitrogen or argon and to addition of small amounts of oxygen are not related to the absence and presence, respectively, of one or more substances exchangeable between yeast cells of different generations in the same way as growth-factors between auxo-heterotrophic organisms in mixed cultures (11, 12, 15). Provided that the cells of the generations developing during the period of cellular adjustment (= initial stationary phase + phase of accelerating growth and multiplication rate) are deficient in one or more specific substances present in the cells produced during later growth periods and causing the resistance of the latter to flushing with oxygen-free nitrogen and argon, then we may conclude that these substances are strongly retained by the cellular structures or at least incapable of penetrating the cytoplasmic membrane and wall of yeast cells under the experimental conditions applied (0.16 M sodium succinate-succinic acid buffer solution, pH 4.9, etc., *cf.* (22, 23, 24)).

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SÉPARATION, PAR CHROMATOGRAPHIE SUR COLONNE DE CELLULOSE, DES SUBSTANCES PHÉNOLIQUES PRODUITES PAR *PENICILLIUM BREVI-COMPACTUM* ET ANALYSE CHIMIQUE SOMMAIRE DE L'UNE D'ENTRE ELLES

par

PAUL GODIN [1])

(Reçu le 1 Mars 1955).

Dans une note antérieure (GODIN 1955), on a signalé que *P. brevi-compactum* (souche Db 182 A) produit onze substances phénoliques différentes: $C_{17}H_{20}O_6$ (acide mycophénolique), $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$, $C_8H_6O_6$ et les substances non identifiées désignées par les chiffres VI, VII, VIII, IX, X et XI. On a indiqué dans cette même note que la moisissure incubant au contact d'une simple solution de glucose substituée au milieu de culture initial après développement complet du microbe, ne produit plus que huit de ces onze substances phénoliques, à savoir: $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$, $C_8H_6O_6$ et les substances non identifiées VI, VII, VIII et IX.

L'objet de la présente communication est de décrire un procédé de séparation de ces différents corps, et notamment des composés non identifiés dont la concentration dans la solution de métabolisme est extrêmement faible.

Les premiers résultats de l'analyse chimique d'une de ces substances sont également exposés ici.

Préparation des solutions à chromatographier.

Seule la solution glucosée substituée au milieu de culture intial est utilisée ici. Ceci permet donc d'éviter la présence dans la solution de métabolisme, de l'acide mycophénolique et des substances phénoliques non identifiées X et XI.

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Après consommation du glucose par la moisissure, la solution nutritive est acidifiée à pH inférieur à 3 par H_2SO_4 dilué. Cette solution est extraite une nuit à l'éther sulfurique dans un extracteur continu. La solution d'extraction est alors évaporée à sec et le résidu est traité suivant la méthode de CLUTTERBUCK et coll. (1932) en vue d'éliminer la plus grande partie de l'acide phénolique le plus abondant: $C_{10}H_{10}O_6$. Le mélange des autres substances phénoliques est dissous dans la quantité minimum du solvant de chromatographie et traité comme décrit ci-après.

Séparation des substances phénoliques.

La méthode utilisée est la chromatographie de partage sur colonne.

Le solvant utilisé est celui qui a été employé avec le plus de succès pour la chromatographie sur papier de ces substances phénoliques (GODIN 1954): un mélange de 5 volumes de chloroforme et de 3 volumes d'acide acétique glacial, additionné de la quantité d'eau tout juste suffisante pour atteindre la saturation sans la dépasser.

On a employé une colonne de poudre de cellulose Whatman, de 4,5 cm de diamètre et de 30 cm de hauteur utile. Le débit de la colonne est réglé à environ 40 cc à l'heure et l'éluat est recueilli, par fraction de 10 cc, au moyen d'un collecteur automatique de fractions.

A chaque opération de chromatographie, on traite 10 cc de la solution saturée du mélange des substances phénoliques dans le solvant de chromatographie. L'élution complète des substances contenues dans ces 10 cc nécessite le passage d'environ 1 litre de solvant à travers la colonne.

Résultats de la chromatographie.

Les substances phénoliques présentes dans chaque fraction de l'éluat sont déterminées par chromatographie sur papier en employant les méthodes décrites dans une note précédente (GODIN 1953).

Les différents composés apparaîssent successivement dans l'éluat, dans l'ordre suivant: la substance IX, la substance VIII, $C_{10}H_{10}O_5$, $C_{10}H_{10}O_7$, $C_{10}H_{10}O_6$, la substance VII, la substance VI et enfin

$C_8H_6O_6$. Ces substances sont accompagnées de diverses impuretés, notamment de matières colorées.

Par cette méthode on parvient, si on opère précautionneusement, à séparer presque quantitativement tous ces composés les uns des autres, sauf la substance VI qui est toujours accompagnée de $C_8H_6O_6$.

La quantité des substances phénoliques non identifiées recueillies à ce jour par ce procédé est encore trop minime pour permettre une analyse chimique complète de ces corps. Leur concentration dans la solution de métabolisme est très faible et la recherche de procédés de purification a fait perdre la plus grande partie de ce qu'on a recueilli.

Seul le composé IX a pu être cristallisé en quantité suffisante pour une analyse sommaire.

Purification et analyse sommaire de la substance phénolique IX.

La fraction de l'éluat renfermant cette substance est fortement teintée en brunâtre par diverses impuretés colorées. Pour s'en débarrasser, on évapore le solvant sous vide jusqu'à sec. Le résidu de cette évaporation est extrait à reflux deux fois de suite, durant une demi-heure, par 100 fois son poids d'éther sulfurique bouillant. Le résidu de ces extractions, un dépôt brun et amorphe, est rejeté.

Du filtrat, on chasse l'éther par distillation. Le dépôt, une huile brunâtre, est repris par 50 fois son poids d'eau bouillante. Après ébullition durant quelques minutes, on se débarrasse par filtration à chaud des gouttelettes huileuses qui surnagent. Par refroidissement, la solution aqueuse laisse déposer une substance amorphe beige-clair. Ce dépôt est dissous dans le minimum d'acétone bouillant et cette solution est versée dans environ 20 fois son volume d'eau distillée bouillante. Par refroidissement, le corps cristallise. On le recristallise dans un mélange d'acétone et d'éther de pétrole.

On obtient ainsi une substance blanche qui fond à 208—212°C. en se décomposant. Très peu soluble dans l'eau froide, modérément soluble à chaud, elle se dissout facilement à froid dans les solutions d'ammoniaque ou des hydroxydes alcalins, mais non dans une solution à 5% de bicarbonate sodique. Laissée au contact de l'air, la solution alcaline jaunit après plusieurs heures. La substance est facilement soluble dans la plupart des solvants organiques, sauf dans l'éther de pétrole où la quantité dissoute est très minime. Les solutions ne présentent aucun pouvoir rotatoire.

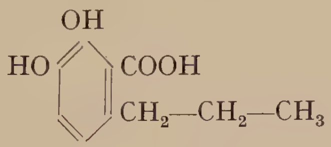
Ce composé ne renferme que C, H et O. La solution saturée dans l'eau donne une teinte rosée peu intense avec le chlorure ferrique et une coloration jaune avec le réactif de Pauly. Elle n'est pas acide à l'égard du tournesol et ne donne aucun précipité avec la 2,4-dinitrophénylhydrazine.

Combustion: $(C_5H_6O_2)n$
Calculé $C = 61{,}21\%$ $H = 6{,}16\%$
Trouvé: $C = 61{,}02\%$ $H = 6{,}05\%$.

L'équivalent de neutralisation de sa solution dans le méthanol est difficilement déterminable. Le point de virage de la phénolphtaléine n'est pas net et les valeurs trouvées oscillent entre 280 et 320.

En bref, il s'agit donc probablement d'un acide phénolique monobasique, non cétonique, de formule $C_{10}H_{12}O_4$.

Rappelons que dans une publication antérieure (GODIN, 1955), on a signalé que le *P. brevi-compactum* étudié produit cette substance, à côté des composés phénoliques VI, VII, VIII, $C_{10}H_{10}O_5$, $C_{10}H_{10}O_7$, et $C_8H_6O_6$, aux dépens de la substance phénolique $C_{10}H_{10}O_6$ lorsque cette dernière est la seule source de carbone mise à la disposition du microbe. Il est donc vraisemblable que $C_{10}H_{12}O_4$ possède le même squelette carboné que $C_{10}H_{10}O_6$, $C_{10}H_{10}O_5$, et $C_{10}H_{10}O_7$. Dans ce cas, il s'agirait probablement du produit de réduction de ces acides phénoliques, le 3,5-dihydroxy, 2-carboxy, propylbenzène,



On notera que chez les composés $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ $(C_{10}H_8O_6—H_2O)$ et $C_8H_6H_6$, le radical correspondant au groupe propyle de $C_{10}H_{12}O_4$ consiste respectivement en: $— CH_2 — CO — CH_3$, $— CHOH — CO — CH_3$, $— CO — CO — CH_3$ et $— COOH$, et que le reste de la molécule est identique à celui de $C_{10}H_{12}O_4$.

Résumé.

Sept des huit substances phénoliques produites par *P. brevi-compactum* aux dépens du glucose seul, ont été séparées par chromatographie sur une colonne de cellulose. L'analyse chimique sommaire de l'une d'entre elles semble indiquer qu'il s'agit du 3,5-dihydroxy, 2-carboxy, propylbenzène: $C_{10}H_{12}O_4$.

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(National Institute of Public Health, Utrecht, Netherlands).

A NEW *SALMONELLA* TYPE (*S. HAARLEM*)

by

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J. H. BEKKER

(Received March 10, 1955).

Some time ago a culture was isolated out of surface water. This culture showed the morphological and biochemical characteristiscs of the genus *Salmonella*, e.g. Gram negative, motile rods, rapid fermentation of glucose (with gas), arabinose, rhamnose, fructose, galactose, threalose, maltose, mannitol, dulcitol and sorbitol, production of H_2S, liquefaction of gelatin, reduction of nitrates. Indol was not formed, urea was not hydrolysed and salicin, inositol, sucrose and lactose were not fermented. Voges-Proskauer test negative, methylred test positive.

The organism was agglutinated to titre by *S. enteritidis* O serum; in absorption tests nearly all the agglutinins were removed from this serum; a serum prepared with the new type agglutinated to the same titre this type and *S. enteritidis*. The agglutinins of this serum were nearly all absorbed by *S. enteritidis*. The culture belonged therefore to group D of the genus *Salmonella*: O antigens 9, 12. The H antigens were diphasic, phase 1 was agglutinated to titre by *S. poona* serum (z) and removed in absorption tests all agglutinins of this serum. Phase 1 of *S. poona* (z) removed in absorption tests all H agglutinins of a serum prepared from phase 1 of the new type. Phase 2 was agglutinated to titre by serum *S. abortus equi* (e, n, x), and absorbed all agglutinins of this serum, while H agglutinins of a phase 2 serum of the new type were all absorbed by *S. abortus equi*.

The antigenic formula of the type was therefore 9,12 : z : e,n,x.

As a *Salmonella* type with this formula, is as yet not described, we propose the name *Salmonella haarlem*.

Summary.

A new *Salmonella* type (*S. haarlem*) with the antigenic formula 9,12 : z : e,n,x, is described.

We are indebted to Miss Dr L. HEMMES (Haarlem) for supplying the water samples and to Dr F. KAUFFMANN, International Salmonella Centre (Copenhagen) for the confirmation of our findings.

(Laboratory of Hygiene, University, Utrecht).

REPRODUCTION OF BACTERIOPHAGE T_6 AND T_7 IN AUXOTROPHIC MUTANTS OF *E. COLI* B

by

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(Received March 14, 1955).

An important part of the protein of bacterial viruses is synthesized from the substrates provided in the medium by the enzymatic apparatus of the host. By using bacterial mutants, auxotrophic for some amino acid, it seemed possible to study whether any amino acid necessary for bacterial growth was also necessary for phage reproduction. Phage infected bacteria of this kind were expected to show phage reproduction in a medium with the auxotrophic factor. Without the amino acid no phage multiplication was expected unless the virus could do without the amino acid.

MATERIALS AND METHODS.

Strains.

Coli bacteriophage T_6 and T_7 were used with as host cells *E. coli* B and a series of mutants obtained with U.V. or X rays by Messrs ROIJERS, VAN DOORN and MULDER in this laboratory. These mutants were all stable. Checks for back mutation were however taken in every experiment.

Strain No.		deficiency	amino acid necessary for half maximal growth
366	U.V.	tryptophane	3 γ/ml
506	U.V.	tyrosine	2 ,,
648	U.V.	serine (glycine)	~40 ,,
411	U.V.	leucine	2.5 ,,
406	U.V.	methionine	2 ,,
429	U.V.	phenylalanine	2.5 ,,
M	X	histidine	2 ,,
M46	X	proline	4 ,,

Media.

Minimal medium (M.M.) for *E. coli* contained per liter: glucose 20 g; KH_2PO_4 6 g; K_2HPO_4 6 g; NH_4Cl 2 g; $MgSO_4$ 7 aq. 0.050 g; $FeSO_4$ 0.0050 g. Sterilized by filtration. Buffer solution as used by BENZER (1952) with or without glucose 20 g. In a series of experiments a mixture containing 18 amino acids in the ratio as they occur in casein was added to the synthetic medium or the buffer solutions. The following quantities in mg/l were used: L-arginine 85; L-histidine 51; L-lysine 160; L-tyrosine 140; DL-tryptophane 56; DL-phenylalanine 210; DL-cystine 12; DL-methionine 142; DL-threonine 166; DL-leucine 400; DL-isoleucine 262; DL-valine 270; glycine 12; DL-alanine 114; DL-serine 302; L-glutamic acid 490; DL-aspartic acid 254; DL-proline 324.

Methods.

Two series of experiments were run, both based on the BENZER technic.

Method A: the bacteria were grown — starting from a large inoculum — for 4 h in aerated M.M. supplemented with the auxotrophic factor. After centrifugation the bacteria were resuspended in buffer solution or in M.M. without ammonium chloride. This suspension was aerated for 10′ and phage was added at a multiplicity of 1 in 10. After adsorption during 15′ the bacteria were spun down, resuspended and diluted 100 and 10.000 fold in the M.M. with or without the auxotrophic amino acid. Free phage was determined with the chloroform method (FREDERICQ, 1950). The growth tubes (100 and 10.000 fold dilutions) were aerated gently and the number of phage particles determined at intervals (ADAMS 1950). The modifications with regard to BENZER's method were necessary as bacteria grown in M.M. easily lose the aptitude to permit phage reproduction in M.M. Thus the washing was omitted and the aeration time reduced.

Method B.

Bacteria were grown as before in M.M. supplemented with 30 γ/ml of the auxotrophic factor. After centrifugation the bacteria were resuspended in buffer supplemented with amino acids but without the auxotrophic factor and aerated for 60′ (unless otherwise stated) to deplete the amino acid pool of the host cell of the auxotrophic amino acid. After centrifugation the cells are resuspended in buffer + glucose infected with phage at low multiplicity (1 in 100) and aerated for 15 min. to permit adsorption. This mixture is then diluted into growth tubes containing M.M. supplemented with 18 amino acids or M.M. with amino acids but without the auxotrophic factor.

RESULTS.

Method A, Phage T_6.

With this method it was not possible to obtain reliable one step growth curves of sufficient step size.

Method A, Phage T_7.

As shown in Fig. 1 T_7-infected cells of a tryptophane-less mutant permit virus multiplication in a synthetic medium with tryptophane. No phage multiplication is observed however if the tryptophane is omitted.

The addition of tryptophane at a later stage produces one step growth curves of lower slopes and lower bursts becoming more and

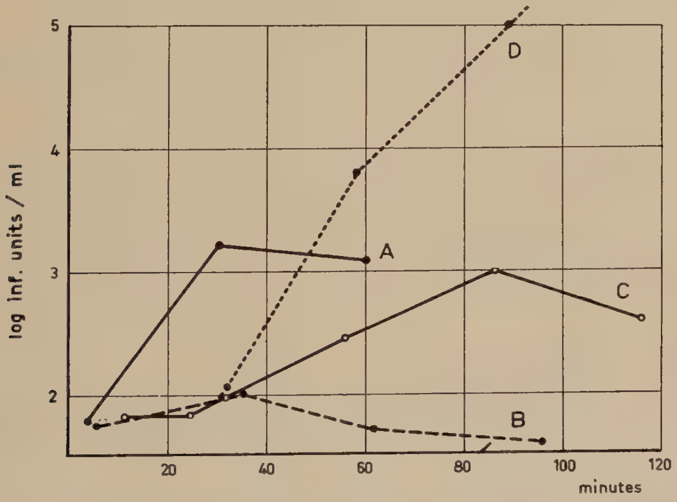

Fig. 1. One step growth curve for phage T_7 in a tryptophane requiring mutant of *E. coli* B in the absence or presence of tryptophane (10 γ/ml). Method A. Response to tryptophane is reduced after 20 minutes though the response to the addition of broth is unimpaired.

Curve A. with tryptophane.
Curve B. without tryptophane.
Curve C. tryptophane added after 20 minutes.
Curve D. Broth added after 20 minutes.

more erratic and irreproducible with time. After forty minutes the infectious units have generally lost the possibility to respond to tryptophane. They still respond to the addition of broth.

Mutant strains of *E. coli* B auxotrophic for tryptophane, tyrosine, leucine, methionine and histidine behave essentially in the same way indicating that bacteriophage T_7 cannot multiply without a supply of these amino acids.

Mutant strains auxotrophic for serine, or for proline showed a different behaviour. In these host cells the multiplication of phage T_7 was equal in the medium with and without serine or proline. These results were not due to traces of the amino acid carried over from the original culture as can be calculated from the dilution scheme. Furthermore washing the bacteria before adsorption, as in the original BENZER technic, did not influence the results though the step size was further reduced.

The phenylalanine-less mutant took an intermediate position in permitting some phage multiplication in media without phenylalanine. The step size was reduced however.

Phage reproduction in auxotrophic mutants without an external supply of the auxotrophic factor might mean that phage reproduction was possible without this amino acid but it seems much more probable that the endogenous supply of amino acids like proline and serine whether in the metabolic pool or in bacterial protein is sufficient for phage synthesis. It was therefore tried to deplete the metabolic pool as described in method B. Furthermore it was thought necessary to study phage T_6 as for this large phage the amount of protein N derived from the medium is much larger.

Method B, Phage T_6.

Phage T_6 reproduced in all mutant strains when the auxotrophic amino acid was present in the growth tube. When the auxotrophic factor was omitted no phage reproduction was observed in the mutants auxotrophic for leucine, tryptophane, tyrosine, phenylalanine, histidine or proline. Fig. 2 gives a representative experiment for a leucine-less mutant.

Fig. 3 shows the influence of aeration time for a proline-auxotrophic mutant. The time necessary for the depletion of the metabolic pool may vary for different amino acids; generally one hour was sufficient.

Phage reproduction was observed however in the mutants auxo-

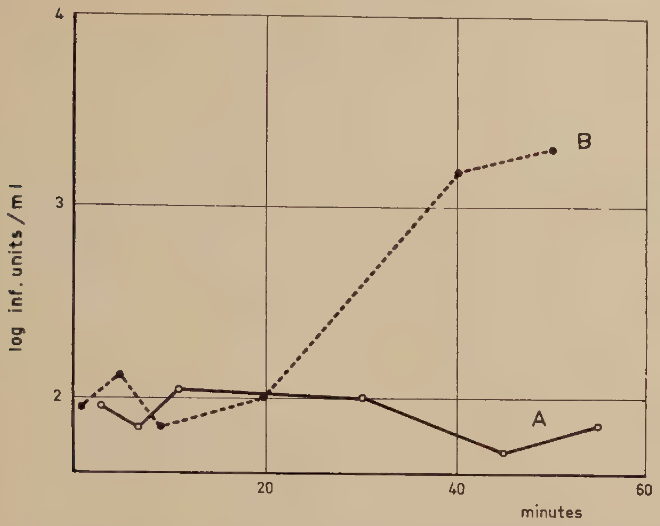

Fig. 2. One step growth curves for phage T_6 in a leucine requiring mutant of *E. coli* B in the absence (A) or presence (B) of leucine (30 γ/ml). Method B.

trophic for phenylalanine, methionine and serine even without the addition of the auxotrophic factors. As the phenylalanine- and methionine-mutants showed some growth in the minimal medium

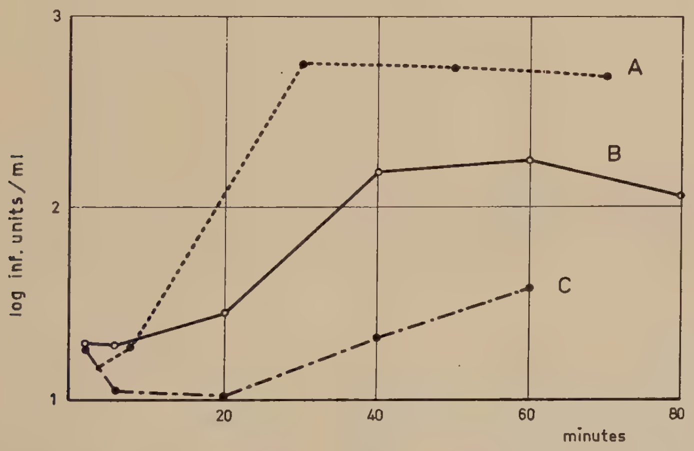

Fig. 3. One step growth curves for phage T_6 in a proline requiring mutant with (A) and without (B and C) proline. Method B.
Curve B. aeration time 15 min.
Curve A and C. aeration time 45 min.
The influence of the depletion of the amino acid pool by aeration is evident.

supplemented with 17 amino acids but without the auxotrophic factor, impurities in the amino acids were suspected.

It could be shown that the tyrosine in the amino acid mixture contained some phenylalanine and that glutamic acid contained methionine. After recristallisation of the tyrosine and purification of the glutamic acid over Amberlite I.R. 4B the experiments were repeated. No phage reproduction in the absence of the auxotrophic factor could now be observed. In relation with these results the minimal concentration of L-methionine which supports phage reproduction was determined (fig. 4).

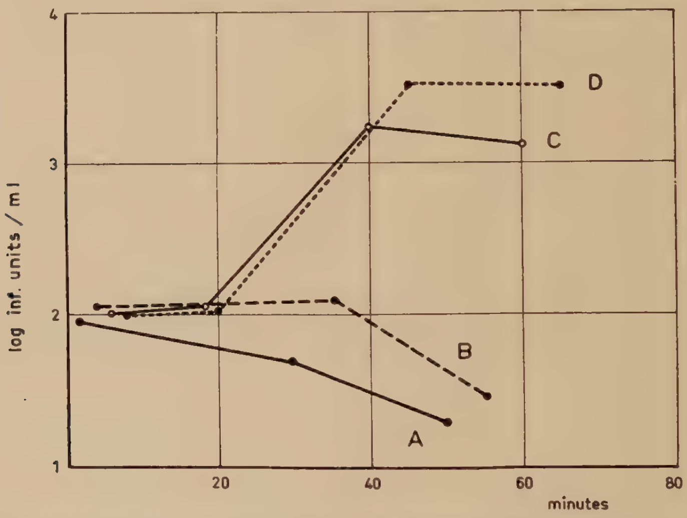

Fig. 4. One step growth curves for phage T_6 in a methionine requiring mutant with and without methionine. Method B.
Curve A. without methionine.
Curve B. with methionine 0.001 γ/ml.
Curve C. with methionine 0.01 γ/ml.
Curve D. with methionine 0.1 γ/ml.

Already 0.01 γ/ml seems to support virus-multiplication whereas the methionine-less *E. coli* showed half maximal growth with 2 γ/ml. The number of bacteria in the growth tubes was about 10^4 per ml (100—200 infectious units). As quantitative data about the growth of the methionine mutant were available (DE HAAN and WINKLER 1955) it could be shown that this small number of bacteria is still able to perform three to four divisions in 0.01γ methionine per ml.

The serine mutant is less apt for this kind of studies as growth was stimulated by glycine, threonine and methionine. Though this strain did not grow on M.M. plus amino acids minus the four substances mentioned it was thought in analogy to the methionine case that very small amounts of impurities in the medium might support phage reproduction. After omission of serine, glycine, threonine, methionine, aspartic acid, glutamic acid and cysteine from the amino acid mixture used for depletion and omission of all amino acids from the phage growth tubes it could be shown that phage did not reproduce unless serine was present.

Method B, Phage T_7.

The results were completely analogous to those with T_6. No phage reproduction was observed in any of the mutants unless the auxotrophic factors were present. For the methionine, phenylalanine and serine mutants, the same precautions as with T_6 had to be taken. Reproduction of phage in proline-mutants without proline-supplement was not observed (as it was with method A). This seems to stress the importance of depleting the amino acid pool.

DISCUSSION.

Neither phage T_6 or T_7 are able to reproduce in the studied mutants of *E. coli* B auxotrophic for an amino acid. Though this does not seem unexpected several features of these experiments seem worth of comment.

a. Depletion of the metabolic pool for the auxotrophic factor is necessary as small reserves in the host cell are able to support phage reproduction.

b. Very small amounts of auxotrophic factor in the medium will support phage multiplication. These amounts are much smaller than those necessary for visible growth of the host bacterium but still sufficient to permit a few divisions of the small number of bacterial cells in the phage growth tubes.

c. The experiments suggest that in these depleted bacteria phage protein is mainly synthesized from the auxotrophic factor in the medium.

d. The experiments suggest that the results of BARNER and COHEN (1954) who found phage reproduction in a thymine-auxotrophic *E. coli* B mutant and concluded that the phage carried a thymine synthetisizing system or a gene controlling thymine synthesis are open to an alternative interpretation.

Summary.

Phage T_6 and T_7 did not reproduce in mutants of *E. coli* auxotrophic for the amino acids methionine, leucine, serine, proline, histidine, tyrosine, phenylalanine and tryptophane unless these amino acids were present in the medium. Very small amounts of these auxotrophic factors (0.01 γ/ml) are able to support phage multiplication. Much care has to be taken to avoid impurities in the medium and to deplete the endogenous reserves of the host cells.

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DE LA SENSIBILITÉ *IN VITRO* DE QUELQUES MICROORGANISMES D'ORIGINE ANIMALE À LA VIOMYCINE

par

J. DEOM et **J. MORTELMANS**

(Reçu le 2 Février 1955).

Depuis la découverte de la Viomycine par FINLAY et collaborateurs (3) à partir de *Streptomyces puniceus* et par BARTZ et collaborateurs (1) à partir de *Streptomyces floridae*, diverses expériences ont été entreprises pour déterminer l'action bactériostatique et bactéricide, aussi bien *in vivo* qu'*in vitro*, de ce nouvel antibiotique sur différents organismes. Citons les travaux d'EHRLICH et collaborateurs (2), de HOBBY et collaborateurs (6) et de LEVADITI et collaborateurs (9). Des essais spéciaux sur *Mycobacterium tuberculosis* ont été réalisés par YOUMANS et STEWART-YOUMANS (11), STEENKEN et WOLINSKY (10), KARLSON et GAINER (7), GERNEZ-RIEUX et collaborateurs (4), GRUMBACH et RIST (5).

En littérature vétérinaire, signalons aussi les expériences *in vitro* sur *Mycobacterium paratuberculosis* par LARSEN et VARDAMAN (8).

A notre tour, nous avons entrepris des essais en vue de déterminer l'action bactériostatique de la Viomycine sur une série de microorganismes isolés de différents animaux malades rencontrés au cours de notre travail de diagnostic au Congo Belge et au Ruanda-Urundi.

Nous avons utilisé dans ces expériences le même milieu de culture pour les microbes à Gram positif et à Gram négatif. Sa composition est la suivante:

Extrait de viande Liebig	3	g
Bacto-peptone Difco	3,3	g
Bacto-tryptose Difco	3,3	g
Peptone pancréatique Boyer	3,3	g
Chlorure sodique	5	g
Eau distillée	1.000	ml

Les ingrédients sont dissouts à chaud dans l'eau distillée. Le pH est amené à 7,4 et la solution est filtrée sur papier filtre ordinaire. Le milieu est réparti en tubes et autoclavé à 120°C. pendant 20 minutes.

Comme antibiotique, nous avons utilisé de la Viomycine présentée sous forme de Viomycin (sulfate) Parke, Davis & Co. en flacons contenant l'équivalent de 1 g de Viomycine base [1]). Pour l'essai, l'antibiotique a été dissout dans de l'eau physiologique (8,5 g de chlorure sodique pour mille) pour obtenir une solution-mère contenant 1 milligramme de Viomycine par millilitre.

On utilise pour l'essai 15 tubes. Le premier contient 3,5 ml du milieu de culture décrit ci-dessus et les 14 autres en contiennent 2 ml. On ajoute aseptiquement au premier tube 0,4 ml de la solution de Viomycine titrée à 1 mg par ml. On mélange et on transporte à la pipette 2 ml dans le second tube. On mélange et on continue ainsi la dilution jusqu'au 14e tube. Le 15e tube ne reçoit pas d'antibiotique et sert de contrôle.

La concentration finale en Viomycine dans ces 15 tubes est donnée au tableau 1.

TABLEAU 1.

Tube	1	2	3	4	5	6	7	8
Microg par ml	100	50	25	12,5	6,25	3,125	1,56	0,78
Arrondi à	100	50	25	12,5	6,25	3,1	1,56	0,8

Tube	9	10	11	12	13	14	15
Microg par ml	0,39	0,19	0,099	0,049	0,024	0,012	0
Arrondi à	0,4	0,2	0,1	0,05	0,025	0,012	0

Les cultures que nous employons comme semence dans ces essais sont préparées de la façon suivante: au moyen d'une anse de platine, on ensemence un bouillon nutritif ordinaire à partir d'une stockculture sur milieu solide et on incube à 37°C. pendant 24

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[1]) Nous remercions la Firme Parke, Davis & Co. de Detroit, Michigan, U.S.A. pour l'obligeance qu'elle a eue de mettre ce nouvel antibiotique à notre disposition. Nous remercions aussi Monsieur le Consul Général de Belgique à New York et Monsieur le Conseiller Vétérinaire du Gouvernement Général du Congo Belge pour leur bienveillante intervention.

TABLEAU 2.

Nombre de souches étudiées	Microorganismes	Concentration inhibitrice minimale en microgrammes par ml
13	Salmonellae groupe B	
	1 souche	plus de 100
	9 souches	100
	1 souche	50
	1 souche	25
	1 souche	1,56
4	Salmonellae groupe C	
	3 souches	100
	1 souche	50
6	Salmonellae groupe D (sauf *S. gallinarum-pullorum*)	
	3 souches	50
	3 souches	25
44	*Salmonella gallinarum-pullorum*	
	1 souche	plus de 100
	2 souches	100
	22 souches	50
	15 souches	25
	2 souches	12,5
	2 souches	6,25
1	*Shigella sonnei*	100
3	*Serratia marcescens*	
	1 souche non-pigmentée	100
	1 souche pigmentée	100
	1 souche pigmentée	50
2	*Proteus vulgaris*	100
1	*Streptococcus zooepidemicus*	12,5
1	*Streptococcus agalactiae*	25
2	*Staphylococcus pyogenes aureus*	25
2	*Staphylococcus pyogenes albus*	12,5
7	*Bacillus anthracis*	
	1 souche	25
	1 souche	12,5
	2 souches	6,25
	2 souches	3,12
	1 souche	1,56
2	*Bacillus subtilis*	
	1 souche	100
	1 souche	50
1	*Erysipelothrix rhusiopathiae*	plus de 100

heures. De cette culture, on prélève une anse de platine de 2 mm de diamètre et on ensemence un tube contenant 2 ml du milieu décrit plus haut. On incube à 37°C. pendant 18 à 20 heures. Cette jeune culture est ensuite diluée au millième dans le même milieu et au moyen d'une pipette graduée on ensemence chaque tube de la série de 15 avec 0,1 ml de la culture diluée. On incube à 37°C. et on lit les résultats après 18 à 20 heures.

Nous désirons attirer ici l'attention sur le fait qu'il est très important d'interpréter les résultats obtenus en regard de la technique employée. Il est d'ailleurs reconnu actuellement par tous les auteurs qui ont travaillé les divers antibiotiques que les résultats peuvent varier suivant la méthode utilisée, le milieu, le temps d'incubation, la quantité de culture, etc.

On trouvera au tableau 2 les valeurs que nous avons obtenues avec les méthodes que nous avons décrites. La concentration en Viomycine qui y est indiquée est celle qui, au moment de la lecture, c'est-à-dire après une incubation de 18 à 20 heures à 37°C., était au minimum indispensable pour inhiber complètement la croissance du microorganisme étudié dans le milieu décrit.

Discussion.

Les auteurs affirment que la Viomycine est douée d'une activité remarquable sur *Mycobacterium tuberculosis*, y compris les souches streptomycino-résistantes, mais seulement d'une activité minime sur les germes non acidorésistants.

Nous avons pu confirmer cette dernière constatation pour des germes isolés de différentes espéces animales à l'exception de *Bacillus anthracis* qui semble aussi être particulièrement sensible à cet antibiotique. Levaditi et collaborateurs (9) avaient déjà attiré l'attention sur ce fait.

Si, à l'instar de ce que font certains auteurs pour quelques autres antibiotiques, on fixe arbitrairement la limite de la sensibilité à 10 microgrammes par ml, on ne trouve que 2 souches de *Salmonella gallinarum-pullorum* sur 42 et une souche de *Salmonella* groupe B qu'on peut considérer comme sensibles. Parmi les 7 souches de *Bacillus anthracis* étudiées, on en trouve 5 sensibles, 1 à la limite de la sensibilité et 1 qu'on peut considérer comme insensible à la Viomycine.

Conclusion.

En appliquant les techniques décrites plus haut à l'étude de la sensibilité à la Viomycine de certains microorganismes isolés d'animaux au Congo Belge et au Ruanda-Urundi, nous avons pu constater ce qui suit:

1°) La plupart des souches de *Bacillus anthracis* sont sensibles à la Viomycine;

2°) Toutes les autres souches examinées, aussi bien à Gram positif qu'à Gram négatif, à l'exception de deux souches de *Salmonella gallinarum-pullorum* et d'une souche de *Salmonella* du groupe B, sont peu ou pas sensibles à la Viomycine.

Summary.

The results of the study of the action of Viomycin on several microorganisms of animal origin isolated in the Belgian Congo and in the Ruanda-Urundi proved that the majority of the strains of *B. anthracis* were inhibited by this antibiotic.

All the other strains under study were not significantly affected by Viomycin with the exception of some strains of *Salmonella*.

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DE LA SENSIBILITÉ *IN VITRO* DE QUELQUES MICROORGANISMES D'ORIGINE ANIMALE À LA NÉOMYCINE

par

J. DEOM et **J. MORTELMANS**

(Reçu le 2 Février 1955).

Depuis la découverte de la Néomycine par Waksman et Lechevalier (16) à partir de *Streptomyces fradiae*, plusieurs expériences ont été entreprises pour déterminer l'action bactériostatique et bactéricide de cet antibiotique, aussi bien *in vivo* qu'*in vitro*, sur toute une gamme de microorganismes d'origine animale (17).

En médecine Vétérinaire, la Néomycine est utilisée jusqu'à présent comme antibiotique d'application locale, notamment dans des cas de dermatites, de mammites, d'entérites, etc. (1—5, 7—15).

Nous avons eu l'occasion d'entreprendre des essais *in vitro* avec la Néomycine en vue de déterminer son action bactériostatique sur une série de microorganismes isolés de différents cas de maladies animales au Congo Belge et au Ruanda-Urundi.

La composition du milieu d'essai et la technique employées sont celles utilisées pour les essais analogues que nous avons réalisés avec la Viomycine (6).

Nous avons employé comme antibiotique la Néomycine, présentée sous la forme de Neomycin Sulfate, Heyden Chemical Corporation [1]).

Nous avons résumé au tableau 1 les valeurs enregistrées avec notre méthode. La concentration indiquée est celle qui, au moment de la lecture, c'est-à-dire après une incubation de 18 à 20 heures

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[1]) Nous remercions la Firme Heyden Chemical Corp. de New York, N.Y., U.S.A. pour l'obligeance qu'elle a eue de mettre ce produit à notre disposition. Nous remercions aussi Monsieur le Consul Général de Belgique à New York et Monsieur le Conseiller Vétérinaire du Gouvernement Général du Congo Belge pour leur bienveillante intervention.

TABLEAU 1.

Nombre de souches étudiées	Microorganismes	Concentration inhibitrice minimale en microgrammes par ml
13	Salmonellae groupe B	
	2 souches	6,25
	4 souches	3,1
	6 souches	1,56
	1 souche	0,4
4	Salmonellae groupe C	
	1 souche	3,1
	2 souches	1,56
	1 souche	0,8
6	Salmonellae groupe D (sauf *S. gallinarum-pullorum*)	
	1 souche	3,1
	2 souches	1,56
	1 souche	0,8
	2 souches	0,4
46	*Salmonella gallinarum-pullorum*	
	5 souches	1,56
	7 souches	0,8
	23 souches	0,4
	7 souches	0,2
	3 souches	0,1
	1 souche	0,025
1	*Shigella sonnei*	3,1
3	*Serratia marcescens*	
	1 souche non pigmentée	1,56
	2 souches pigmentées	0,8
2	*Proteus vulgaris*	3,1
1	*Streptococcus zooepidemicus*	0,4
1	*Streptococcus agalactiae*	0,4
2	*Staphylococcus pyogenes aureus*	0,012
2	*Staphylococcus pyogenes albus*	0,012
7	*Bacillus anthracis*	
	1 souche	0,1
	3 souches	0,05
	3 souches	0,025
2	*Bacillus subtilis*	
	1 souche	0,8
	1 souche	0,1
1	*Erysipelothrix rhusiopathiae*	25

à 37°C., était au minimum indispensable pour inhiber complètement la croissance du microorganisme étudié dans le milieu décrit.

Conclusion.

Avec la technique employée, toutes les souches de microorganismes isolés d'animaux au Congo Belge et au Ruanda-Urundi et que nous avons étudiées, se sont révélées sénsibles *in vitro*, à une exception près, à la Néomycine.

Summary.

All the microorganisms of animal origin, isolated in the Belgian Congo and in the Ruanda-Urundi and tested for sensibility to Neomycin, were inhibited by this antibiotic, with the exception of one strain of *Erysipelothrix rhusiopathiae*.

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CHEMICAL AND BIOLOGICAL STUDIES ON 1,2-DIHYDRO-*S*-TRIAZINES

VII. ACTIVITY IN PTEROYLGLUTAMIC ACID-*LACTOBACILLUS CASEI* # 7469 SYSTEMS [1]

by

G. E. FOLEY and **E. C. HALEY** [2]

(Received March 30, 1955).

INTRODUCTION.

Studies on the nature of sulfonamide inhibition (15, 23, 35, 38) and the suggestion offered by FILDES (6), that useful chemotherapeutic agents might be realized by the structural alteration of some specific metabolite essential to various pathogenic microörganisms, renewed interest in EHRLICH's concept of the chemotherapy of infectious disease. This direction of research, further stimulated by the advent of antibiotics, has resulted in innumerable studies concerned in one way or another with the fundamental biochemistry of cell growth, and the consequent evolution of present-day concepts of specific metabolite-antimetabolite interrelationships (39, 40). The application of these principles to other biological problems, particularly that of malignancy, for some time lagged behind developments in the field of infectious disease. The identification, isolation and delineation of the haematopoietic effect of pteroylglutamic acid (PGA) (see 20, 21 for review) followed by the successful use of structural analogues of this essential growth factor which were designed specifically to produce the opposite haematopoietic effect ascribed to PGA, in the chemotherapy of the acute leukemias of childhood by FARBER and his associates (3) provided the stimulus for intensive research in the chemotherapy of malignant disease.

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[1] Part I: J. Amer. Chem. Soc. **74**, 855, 1952; Amer. J. Path. **28**, 599, 1952; II: J. Amer. Chem. Soc. 1955, in press; III: J. Amer. Chem. Soc. 1955, in press; IV: Proc. Soc. Exp. Biol. Med. **83**, 733, 1953; V: Proc. Soc. Exp. Biol. Med. **83**, 740, 1953; VI: Proc. Soc. Exp. Biol. Med. **83**, 742, 1953.

[2] The experimental work for this and studies VIII, IX, and X in this series was done in part at the Laboratorium voor de Gezondheidsleer, Universiteit van Amsterdam, Nederland, which is directed by Professor Dr A. CHARLOTTE RUYS, and these four reports are based upon a thesis submitted by G.E.F. to the Faculteit der Wis- en Natuurkunde, in partial fulfilment of the requirements for the degree of Doctor of Science.

The discovery and study of the 1,2-dihydro-*s*-triazines represents a part of a broad program under the over-all direction of Dr SIDNEY FARBER in the chemotherapy of malignancy in an institution devoted to the study of the biological problems relating to malignancy. The synthesis of the first member of this hitherto undescribed class of compounds was accomplished in 1949 by MODEST (24) in these laboratories during the course of a program of synthesis of a series of arylbiguanides. The new compound was produced by the condensation of ethyl-*p*-aminobenzoate, dicyandiamide and concentrated hydrochloric acid in acetone (24, 25). The structure 4,6-diamino-1-(*p*-carbethoxyphenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride was assigned to this compound (24, 25, 28). The reaction proved to be general for the synthesis of a series of 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines and was extended to include aldehydes as well as ketones (28). An alternate method of synthesis by the condensation of arylbiguanides with ketones or aldehydes under acid conditions also has been developed (29).

The original observations on the biological activity of this family of compounds also were made in these laboratories early in 1950 with the parent compound of the series (24). Since that time, more than 275 samples of 85 derivatives have been studied extensively in microbiological and other bioassay systems.

These compounds, like the structural analogues of PGA, exhibit activity in several *in vivo* biological systems, *e.g.*, experimental tumors (4, 24, 25), experimental malaria (5, 16, 24), experimental coccidiosis (22) and murine toxoplasmosis (12, 37), as well as *in vitro* in microbiological (7-12, 24, 26, 27), mammalian liver (10, 12, 13) and botanical (32) bioassay systems. Previous communications have recorded the non-competitive inhibition of PGA in *Streptococcus faecalis* # 8043 ¹) bioassay systems by certain of these com-

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¹) American Type Culture Collection numbers.

pounds (7, 8, 24) and the reversal of this inhibition by the metabolic derivatives of PGA (7, 8, 11, 24).

The present report is concerned with the study of these inhibitors in *Lactobacillus casei* # 7469 [1]) — PGA bioassay systems. Subsequent papers in this series will record the results of additional studies in other microbiological systems, together with observations on the mechanism of action by which the 1,2-dihydro-*s*-triazines inhibit the utilization of PGA.

METHODS.

PGA bioassays using *Lactobacillus casei* were done in the medium described by TEPLY and ELVEHJEM (36). Methods of preparation and bioassay otherwise were as previously described (8, 9, 12). The dihydrotriazine hydrochlorides characterized by a 50% inhibition index of less than 100 vs PGA (8) in *Streptococcus faecalis* bioassay systems were used as the basis for these studies. These compounds were titrated vs PGA in *Lactobacillus casei* bioassay systems and the 50% inhibition indices (19) computed.

RESULTS.

The 50% inhibition indices obtained with 2,2-di-substituted and 2-mono-substituted derivatives are presented in Table 1. As was observed in *Streptococcus faecalis*-PGA bioassay systems (8, 9) microbiological activity varied considerably with substitution in the molecule. In general, the 2,2-dimethyl-($R_2 = R_3 = CH_3$) substituted derivatives exhibited greater activity than the corresponding isomeric and homologous derivatives, as illustrated by comparison of the microbiological activity of D–54 . HCl [2]) ($R_2 = R_3 = CH_3$) with that of D–63 . HCl ($R_2 = C_2H_5$, $R_3 = H$), D–67 . HCl ($R_2 = CH_3$, $R_3 = H$) and D–75 . HCl ($R_2 = CH_3$, $R_3 = C_2H_5$) (Table 1). Maximum activity was obtained with the 2,2-dimethyl substituted derivative in which the phenyl ring is unaltered (D–23 . HCl) or contains a halogen substituent in the *meta*-position (D–69 . HCl), while activity was decreased somewhat by *para*- (D–20 . HCl) or *para*- and *meta*-halogen substitution in the phenyl ring, *e.g.*, D–54 . HCl, Table 1.

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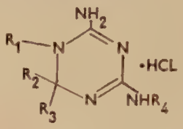
[1]) American Type Culture Collection numbers.

[2]) Two samples of this compound were used, one synthesized in these laboratories by Dr E. J. MODEST and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

TABLE 1.

Inhibition of *Lactobacillus casei* ≠ 7469 — PGA bioassay systems by ketone and aldehyde derivatives of the 1,2-dihydro-*s*-triazines.

Structure 1)



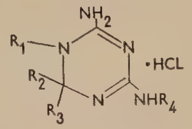
	Structure 1)			50% Inhibition Index vs PGA gamma/ml	
	R_1	R_2	R_3	0.001	0.0001
Ketone derivatives					
D–69	3′-chlorophenyl	methyl	methyl	6,000	6,500
D–23(3,5)	phenyl	,,	,,	9,000	9,400
D–54(3,6)	3′, 4′-dichlorophenyl	,,	,,	11,000	11,000
D–20(2,3,4,5)	4′-chlorophenyl	,,	,,	19,500	18,000
D–65	4′-bromophenyl	,,	,,	27,000	27,000
D–83	4′-tolyl	,,	,,	38,000	38,000
D–19(2)	4′-carbethoxyphenyl	,,	,,	80,000	80,000
D–75	3′, 4′-dichlorophenyl	,,	ethyl	160,000	162,000
D–32(1)	phenyl	,,	methyl	300,000	300,000
Aldehyde derivatives					
D–84	4′-chlorophenyl	*n*-hexyl	H	9,600	9,700
D–67	3′, 4′-dichlorophenyl	methyl	H	32,000	32,000
D–76	3′, 4′-dichlorophenyl	*n*-propyl	H	50,000	46,000
D–63(3)	3′, 4′-dichlorophenyl	ethyl	H	54,000	54,000
D–55	3′, 4′-dichlorophenyl	phenyl	H	120,000	120,000

1) R_4 = H except in case of compound D-32.HCl, where $R_4 = CH_3$.
2) Free base microbiologically active.
3) Dihydrochloride microbiologically active.
4) Nitrate, nitrite and bicarbonate microbiologically active.
5) Picrate microbiologically active.
6) Sample received through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England, showed similar microbiological activity.

n-Propyl substitution at the 2-position of the triazine ring resulted in a reduction of activity in either the *para*- or *para*- and *meta*-halogen substituted derivatives, as indicated by comparison of the microbiological activity of D–20 . HCl and D–54 . HCl with that of D–48 . HCl and D–76 . HCl, respectively (Tables 1 and 2); activity was restored by *n*-hexyl substitution at the same position *e.g.*, D–84 . HCl (Table 1). These variations in activity are similar to those observed with these derivatives in *Streptococcus faecalis* and *Leuconostoc citrovorum* bioassay systems (8, 9), as indicated

TABLE 2.

Effect of substitution on activity of dihydrotriazine hydrochlorides in microbiological systems.

	Structure 				50% Inhibition Index		
	R_1	R_2	R_3	R_4	*Streptococcus faecalis* # 8043 PGA, 0.001 gamma/ml	*Lactobacillus casei* # 7469 PGA, 0.001 gamma/ml	*Leuconostoc citrovorum* # 8081 CF, 0.001 gamma/ml
D-22 . HCl	4′-carboxyphenyl	methyl	methyl	H	3,500,000	Inactive [1]	Inactive [1]
D-23 . HCl	phenyl	methyl	methyl	H	110	9,000	9,200
D-32 . HCl	phenyl	methyl	methyl	methyl	114	300,000	Inactive [1]
D-68 . HCl	2′-chlorophenyl	methyl	methyl	H	72,400	200,000	640,000
D-20 . HCl	4′-chlorophenyl	methyl	methyl	H	95	19,500	20,000
D-48 . HCl	4′-chlorophenyl	*n*-propyl	H	H	1,750	480,000	500,000
D-84 . HCl	4′-chlorophenyl	*n*-hexyl	H	H	220	9,600	116,000

1) at 1.0 mg/ml.

in Table 2. Similarly, substitution of a sufficiently large blocking group, either at the *ortho*- position of the phenyl ring (*e.g.*, D–68 . HCl, Table 2) or at the 2-position of the triazine ring, has the same effect, *i.e.*, reduction of microbiological activity, as was observed in other bioassay systems (Table 2). Methylation of the 4-amino group of the triazine ring of the 2,2-dimethyl-phenyl derivative resulted in a marked decrease in activity, as illustrated by comparison of D–23 . HCl and D–32 . HCl. This alteration in the molecule did not produce a marked change in activity in *Streptococcus faecalis* bioassay systems, but did result in the loss of activity vs citrovorum factor (CF) in *Leuconostoc citrovorum* bioassay systems (Table 2). It is of interest to note that the *para*-carboxyphenyl-2,2-dimethyl substituted derivative (D–22 . HCl), the only otherwise unaltered dihydrotriazine hydrochloride which was inert in *Streptococcus faecalis* bioassay systems, also was inactive vs *Lactobacillus casei* (Table 2). The free bases (25, 28) also were active (Table 1), while a number of the isomeric (DX) compounds (24, 25, 28) exhibited considerable activity vs *Lactobacillus casei* (Table 3), in contrast to their relative inactivity in *Streptococcus faecalis* bioassay systems (8, 9).

Inspection of the 50% inhibition indices (Table 1) indicates that these compounds exhibit a competitive inhibition of PGA in *Lactobacillus casei* bioassay systems. The range of metabolite concentrations over which a competitive relationship with PGA obtains varies with different derivatives and disappears in the presence of high concentrations of metabolite, as illustrated in Table 4. However, the range of competitive inhibition is greater than that observed in *Streptococcus faecalis* bioassay systems (8, 9). Changes in the inhibition index with increasing concentrations of metabolite similar to those noted here have been reported with 4-aminopteroylglutamic acid ¹) (4-APGA) (31).

Inhibition analyses (34) indicated that inhibition by the dihydrotriazine hydrochlorides in *Lactobacillus casei* bioassay systems may be reversed by appropriate concentrations of dihydropteroylglutamic acid ¹) (dihydro-PGA) (30), N^{10}-formylpteroylglutamic acid ¹) (N^{10}-formyl-PGA) (14), leucovorin ¹) (1) and (CF) ¹) (33).

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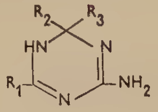
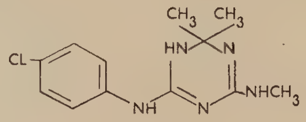
¹) We wish to express our appreciation to the Lederle Laboratories, Pearl River, New York, for generous samples of these compounds. Another sample of dihydropteroylglutamic acid, synthesized in these laboratories by Dr E. J. Modest according to ref. (30), also was used in these studies.

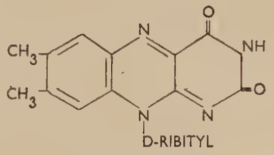
TABLE 3.

Activity of certain anilinodihydrotriazines in PGA bioassay systems.

Compound	Structure [1] 			50% Inhibition Index vs PGA, 0.001 gamma/ml	
	R_1	R_2	R_3	*Lactobacillus casei*, # 7469	*Streptococcus faecalis*, # 8043
DX–19	4′-carbethoxyanilino	methyl	methyl	190,000	980,000
DX–20	4′-chloroanilino	methyl	methyl	60,000	52,000
DX–22	4′-carboxyanilino	methyl	methyl	1,000,000	1,130,000
DX–29 [1]	anilino	methyl	methyl	840,000	750,000
DX–34	anilino	H	phenyl	620	1,560,000
DX–35 . HCl [1]	4′-chloroanilino	methyl	methyl	550	200,000
DX–60	4′-chloroanilino	H	ethyl	610	500,000



DX–35



Riboflavin

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[1] In compound DX–29, NH_2 is replaced by $N(CH_3)_2$; in compound DX–35, NH_2 is replaced by $NHCH_3$.

TABLE 4.

Effect of increasing concentrations of metabolite on inhibition of *Lactobacillus casei* # 7469 bioassay systems by dihydrotriazine hydrochlorides.

	50% Inhibition Index vs PGA, gamma/ml				
Compound [1]	1.0	0.1	0.01	0.001	0.0001
D–69 . HCl	58	580	5,800	6,000	6,500
D–54 . HCl	48	480	4,800	11,000	11,000
D–20 . HCl	56	560	5,600	19,500	18,000
D–84 . HCl	60	600	6,000	9,600	9,700

[1]) See Table 1 for chemical structure.

Inhibition also is reversed partially by excess uracil and uridylic acid, desoxyribonuleic acid and more completely by thymine and thymidine, but not by adenine or guanine, as was observed in *Streptococcus faecalis* bioassay systems (8, 9). Reversal of the inhibition of *Lactobacillus casei* differed however, in that inhibition was reversed by excess PGA over a much broader range of inhibitor concentration, *i.e.*, the range of competitive inhibition in either bioassay system, yet inhibition in *Lactobacillus casei*, like that in *Streptococcus faecalis* bioassay systems (8, 9,) was irreversible with PGA beyond the competitive range of metabolite-inhibitor concentrations.

The inhibition of *Lactobacillus casei* bioassay systems by certain of the DX structures (Table 3) was reversed more readily by high concentrations of riboflavin than by the metabolic derivatives of PGA. Inhibition was essentially non-competitive with riboflavin, and in this respect resembled the anti-riboflavin activity exhibited by certain other compounds, particularly 2-*p*-chloroanilino-4-*β*-dimethylaminoethylamino-6-methylpyrimidine (2), which bear a configurational resemblance to riboflavin, as do the DX compounds (Table 3).

Dihydro-PGA, N^{10}-formyl-PGA, and CF were inhibited by the dihydrotriazine hydrochlorides when substituted for PGA in this bioassay system. The inhibition of these metabolites appeared to be competitive only over a narrow range of concentration of metabolite, and the similarity of the various inhibition indices to one another and to PGA was in sharp contrast to the differences exhibited in *Streptococcus faecalis* bioassay systems (8, 9). The inhibition indices obtained vs PGA with *Lactobacillus casei* resemble

TABLE 5.

Comparison of activity of dihydrotriazines in microbiological systems.

Compound [1]	50% Inhibition Index *Streptococcus faecalis* # 8043 (24 hrs.) PGA	 CF	 *Lactobacillus casei* # 7469 (48 hrs.) PGA	 CF	 *Leuconostoc citrovorum* # 8081 (48 hrs.) CF
	0.001 gamma/ml		0.001 gamma/ml		0.001 gamma/ml
D–69 . HCl	75	2,000	6,000	8,200	9,370
D–23 . HCl	110	2,500	9,000	8,400	9,200
D–54 . HCl	35	11,600	11,000	40,000	22,000
D–20 . HCl	95	10,500	19,500	60,000	20,000
D–84 . HCl	220	48,000	9,600	30,000	116,000

[1] See Table 1 for chemical structure.

those obtained vs CF in the other bioassay systems, rather than the lower indices obtained vs PGA with *Streptococcus faecalis* (8, 9) (Table 5), suggesting a different mechanism of inhibition. This difference is further emphasized by comparison of the inhibition indices obtained vs CF in three microbiological systems with those vs PGA and CF in *Streptococcus faecalis* bioassay systems, since there is a general correlation in order of magnitude among the inhibition indices obtained vs CF with *Lactobacillus casei, Streptococcus faecalis* and *Leuconostoc citrovorum* # 8081 (Table 5).

Discussion.

The microbiological activity of the dihydrotriazine hydrochlorides in *Lactobacillus casei* resembles that exhibited in *Streptococcus faecalis* bioassay systems (8, 9) in that the nature of the metabolite-inhibitor relationship changes with increasing concentrations of metabolite. However, competitive inhibition obtains over a much wider range of metabolite concentrations with *Lactobacillus casei* than with *Streptococcus faecalis*, hence the results with *Lactobacillus casei* are considered to represent a competitive inhibition similar to that exhibited by 4-APGA. Similar results, *i.e.*, non-competitive inhibition of *Streptococcus faecalis* and competitive inhibition of *Lactobacillus casei*, have been reported with several classes of compounds bearing a formal structural relationship to these compounds, such as the aryl-diamino-pyrimidines (17, 18), -*as*-triazines (18) and -triazoles (18). The inhibition of either bioassay system, however,differs from that induced by the usual structural analogues of PGA in that inhibition is not relieved by adenine or guanine and, at appropriate concentrations of inhibitor, is not reversed by PGA.

Inhibition analyses suggest that the mechanism of inhibition of *Lactobacillus casei* may differ from that proposed for *Streptococcus faecalis* (8, 9). The 2,2-dimethyl-phenyl and the 2,2-dimethyl-*m*-chlorophenyl derivatives which were the most active vs PGA and CF in *Lactobacillus casei* bioassay systems also exhibited greatest activity vs dihydro-PGA, N^{10}-formyl-PGA and CF in *Streptococcus faecalis* and vs CF in *Leuconostoc citrovorum* bioassay systems (8, 9). These observations, together with the general correlation between activity vs PGA with *Lactobacillus casei* and activity vs CF in three microbiological systems as presented here, suggest that dihydrotriazine hydrochloride inhibition in *Lactobacillus casei* bioassay systems may be the result of interference with the utilization, rather than inhibition of the biosynthesis of CF.

Summary.

A series of 1,2-dihydro-*s*-triazines has been studied in *Lactobacillus casei* ♯ 7469-pteroylglutamic acid systems. The active derivatives exhibit a competitive inhibition similar to that of 4-aminopteroylglutamic acid, but differ from the latter in that inhibition is not relieved by adenine or guanine, and at appropriate concentrations of inhibitor, is not reversed by excess pteroylglutamic acid. Differences in microbiological activity can be correlated with certain alterations in the structure of the molecule, maximum activity being exhibited by the 2,2-dimethyl-phenyl- and 2,2-dimethyl-*m*-chlorophenyl derivatives.

The inhibitory effect of these compounds is reversed by appropriate concentrations of dihydropteroylglutamic acid, N^{10}-formylpteroylglutamic acid, synthetic and natural citrovorum factor, thymine and thymidine. The similarity in inhibition indices obtained vs the various forms of pteroylglutamic acid in *Lactobacillus casei* bioassay systems and the correlation with those vs citrovorum factor in *Streptococcus faecalis* ♯ 8043 and *Leuconostoc citrovorum* ♯ 8081 bioassay systems suggests that inhibition of *Lactobacillus casei* is the result of interference with the utilization of citrovorum factor.

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(From the Laboratories of Microbiology, The Children's Cancer Research Foundation, and the Department of Pathology, Harvard Medical School, at The Children's Medical Center, Boston, Massachusetts, U.S.A.).

CHEMICAL AND BIOLOGICAL STUDIES ON 1,2-DIHYDRO-*S*-TRIAZINES

VIII. ACTIVITY IN NICOTINIC ACID, RIBOFLAVIN AND THIAMINE SYSTEMS [1]

by

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(Received March 30, 1955).

INTRODUCTION.

Previous reports have recorded the inhibition of pteroylglutamic acid (PGA) in *Streptococcus faecalis* # 8043 [2] (6) and *Lactobacillus casei* # 7469 [2] bioassay systems (7) by a series of symmetrical 1,2-dihydro-*s*-triazines (13, 14) and the reversal of this inhibition by certain metabolic derivatives of PGA (6, 7). The present communication is concerned with the study of a number of these inhibitors in riboflavin, thiamine and nicotinic acid bioassay systems.

METHODS.

Lactobacillus arabinosus # 8014 [2] — nicotinic acid, *Lactobacillus casei* # 7469 [2] — riboflavin and *Lactobacillus fermenti* # 9338 [2] — thiamine bioassays were done in the respective media as prepared by the Difco Laboratories, Inc. Methods of preparation and bioassay otherwise were as previously described (6, 7). The various dihydrotriazine derivatives were titrated in these media and the 50% inhibition indices (10) computed.

RESULTS.

***Lactobacillus arabinosus* - nicotinic acid bioassay systems.**

The phenyl, *para*-chloro-, bromo-, methyl-, carbethoxyphenyl, and *meta*-chlorophenyl derivatives with 2,2-dimethyl-substitution were equally active, while 3′, 4′-dichloro-substitution in the phenyl ring produced a marked increase in activity, as illustrated by comparison of D–20 · HCl and D–54 · HCl [1]) (Table 1). The 2,2-dimethyl ($R_2 = R_3 = CH_3$) substituted derivatives in general exhibited greater activity than the corresponding isomeric and homologous derivatives, as illustrated by comparison of the microbiological activity of D–54 · HCl ($R_2{=}R_3{=}CH_3$) with that of D–75 · HCl ($R_2{=}CH_3$, $R_3{=}C_2H_5$) and D–67 · HCl ($R_2{=}CH_3$, $R_3{=}H$) (Table 1). Substitution of a sufficiently large group at the *ortho*- position of the phenyl ring (D68 · HCl, Table 1) resulted in reduced microbiological activity, as was observed in other microbiological systems (6, 7). Variations in activity resulting from increasing size of the substituent at the 2-position of the triazine ring also paralleled those observed in other bioassay systems (6, 7) (Table 2). The free bases (13) also exhibited microbiological activity, while the isomeric (DX) compounds (13, 14) again were less active than the corresponding (D · HCl) structures, as was observed in *Streptococcus faecalis* bioassay systems (6).

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[1] Part VII: Antonie van Leeuwenhoek 21, 385, 1955.

[2] American Type Culture Collection numbers.

Inhibition analyses indicate that inhibition in *Lactobacillus arabinosus* bioassay systems may be reversed non-competitively by appropriate concentrations of dihydropteroylglutamic acid [2]) (dihydro-PGA) (15), N^{10}-formylpteroylglutamic acid [2]) (N^{10}-formyl-PGA) (8), leucovorin [2]) (1) and citrovorum factor [2]) (CF) (18), and is partially reversed by excess uracil or uridylic acid, desoxyribonucleic acid and more completely by thymine or thymidine but not by PGA, as was observed with *Streptococcus faecalis* (6) and *Lactobacillus casei* (7). The inhibition of *Lactobacillus arabinosus* also may be reversed by high concentrations of nicotinic acid (circa 0.2 mg/ml) as observed earlier in *Streptococcus faecalis* — PGA bioassay systems (7) (Table 3). Since nicotinic acid exists largely as coenzyme I (19) in this microörganism (12), purified coenzyme

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[1]) Two samples of this compound were used, one synthesized in these laboratories by Dr E. J. Modest and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

[2]) We wish to express our appreciation to the Lederle Laboratories, Pearl River, New York, for generous samples of these compounds. Another sample of dihydropteroylglutamic acid, presumably the 7,8-dihydro derivative (15), synthesized in these laboratories by Dr E. J. Modest, also was used in these studies.

TABLE 1.

Activity of dihydrotriazine hydrochlorides in *Lactobacillus* bioassay systems.

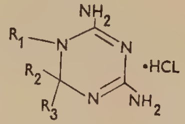
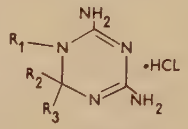
Compound	Structure			50% Inhibition Index		
				*Lactobacillus arabinosus* # 8014 Nicotinic Acid, 0.01 gamma/ml	*Lactobacillus casei* # 7469 Riboflavin, 0.01 gamma/ml	*Lactobacillus fermenti* # 9338 Thiamine, 0.01 gamma/ml
	R_1	R_2	R_3			
D–23	phenyl	methyl	methyl	1,000	100,000	100,000
D–20	4′-chlorophenyl	,,	,,	1,000	100,000	100,000
D–54	3′-4′-dichlorophenyl	,,	,,	50	50,000	100,000
D–75	3′, 4′-dichlorophenyl	,,	ethyl	10,000	10,000	50,000
D–67	3′, 4′-dichlorophenyl	,,	H	10,000	10,000	50,000
D–68	2′-chlorophenyl	,,	methyl	20,000	100,000	100,000

TABLE 2.

Effect of variations at 2-position of triazine ring on microbiological activity of dihydrotriazine hydrochlorides.

	Structure			50% Inhibition Index		
	 			*Lactobacillus arabinosus* # 8014 Nicotinic Acid, 0.01 gamma/ml	*Streptococcus faecalis* # 8043 PGA, 0.01 gamma/ml	*Leuconostoc citrovorum* # 8081 CF, 0.00005 gamma/ml
Compound	R_1	R_2	R_3			
D–54	3′, 4′-dichlorophenyl	methyl	methyl	50	10	11,600
D–63	3′, 4′-dichlorophenyl	ethyl	H	1,000	40	126,000
D–75	3′, 4′-dichlorophenyl	methyl	ethyl	10,000	37.5	187,000
D–76	3′, 4′-dichlorophenyl	*n*-propyl	H	10,000	95	200,000
D–55	3′, 4′-dichlorophenyl	phenyl	H	5,000	62	172,000
D–84	4′-chlorophenyl	*n*-hexyl	H	1,000	45	76,000

I [1]) was studied in this bioassay system. Coenzyme I proved to be equally as active as nicotinic acid or nicotinamide with respect to supporting the growth of *Lactobacillus arabinosus* and the reversal of dihydrotriazine hydrochloride inhibition (Table 3). Coenzyme I also reversed inhibition in *Streptococcus faecalis* — PGA and *Lactobacillus casei* — riboflavin bioassay systems, although somewhat less effectively (Table 3).

The inhibition of *Lactobacillus arabinosus* differs from that of *Streptococcus faecalis* (6) in that high concentrations of *para*-aminobenzoic acid (PABA) also reverse inhibition (Table 3). Since this microörganism is known to synthesize PGA (16, 17) and CF (9) from PABA; the reversibility of inhibition by PABA suggested that the dihydrotriazine hydrochlorides also might inhibit this conversion. Preliminary studies utilizing the *Lactobacillus arabinosus* — PABA bioassay system described by LEWIS (11) indicate that high concentrations of certain derivatives of this series do inhibit the response of *Lactobacillus arabinosus* to PABA.

***Lactobacillus casei* - riboflavin bioassay systems.**

The dihydrotriazine hydrochlorides and the corresponding free bases were relatively poor inhibitors, with inhibition indices ranging from 10,000 (D–75 · HCl) to 100,000 (D–20 · HCl) or more in the presence of 0.01 gamma/ml of riboflavin (Table 1). Inhibition in this bioassay system was reversed non-competitively by excess riboflavin and with difficulty by high levels of the various derivatives of PGA, probably because of the high concentrations of the dihydrotriazine derivatives required to inhibit the system. Inhibition was not reversed by PGA or PABA, but was partially reversed by nicotinic acid or coenzyme I (Table 3). Certain of the DX structures, DX–34, DX–35 · HCl and DX–60 (13, 14), exhibited activity comparable to that observed in *Lactobacillus casei* — PGA bioassay systems and were similarly reversed by high levels of riboflavin (7).

***Lactobacillus fermenti* - thiamine bioassay systems.**

All of the dihydrotriazine derivatives studied were relatively poor inhibitors, with inhibition indices ranging from 50,000 to 100,000 or more in the presence of 0.01 gamma/ml of thiamine

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[1]) Product of the Sigma Chemical Company, St. Louis, Missouri.

TABLE 3.

Comparison of reversibility of dihydrotriazine hydrochloride inhibition in different bioassay systems.

		Lactobacillus arabinosus # 8014 Nicotinic Acid, 0.01 gamma/ml + D-54 . HCl, 5.0 gamma/ml	*Lactobacillus casei* # 7469 Riboflavin, 0.01 gamma/ml + D-54 . HCl, 400 gamma/ml	*Streptococcus faecalis* # 8043 PGA, 0.01 gamma/ml + D-54 . HCl, 0.6 gamma/ml
Metabolite	gamma/ml	Optical density × 10		
Nicotinic acid	200	7.00	2.80	3.00
Nicotinamide	200	7.00	0.00	0.00
Riboflavin	200	0.00	4.00	0.00
Thiamine	200	0.00	0.00	0.00
Pantothenate	200	0.00	0.00	0.00
Biotin	1	0.00	0.00	0.00
Coenzyme I	100	7.00	2.40	2.20
para-Aminobenzoic acid	1200	1.80	0.00	0.00
	600	3.50	0.00	0.00
	300	4.80	0.00	0.00
	100	4.20	0.00	0.00
Controls				
None	—	0.00	0.00	0.00
Nicotinic acid, 0.01 gamma/ml alone		6.60	—	—
Riboflavin, 0.01 gamma/ml alone		—	5.60	—
PGA, 0.01 gamma/ml alone		—	—	5.20

(Table 1). The results of inhibition analyses were similar to those described for *Lactobacillus casei* — riboflavin bioassay systems.

DISCUSSION.

These studies indicate that the dihydrotriazine hydrochlorides are relatively poor inhibitors of riboflavin and thiamine in these bacterial bioassay systems. The slight anti-riboflavin activity exhibited by these compounds perhaps is attributable to their vague configurational resemblance to riboflavin as suggested previously (7); likewise, the increased activity of certain anilino (DX) derivatives in *Lactobacillus casei* — riboflavin bioassay systems may be the result of a configurational resemblance to riboflavin, as may be the case with certain 2-anilino pyrimidines which also exhibit anti-riboflavin activity (2). The reversibility of inhibition in either bioassay system by high concentrations of the metabolic derivatives of PGA suggests that these inhibitions also are concerned with the metabolism of PGA. The results of studies in *Lactobacillus arabinosus* — nicotinic acid bioassay systems are of particular interest in that the dihydrotriazine hydrochlorides appear to inhibit the utilization of PABA. Similar observations with a series of pteridine derivatives which are antagonists of PGA have been reported (3, 4, 5).

The reversal of inhibition by coenzyme I also is of particular interest, in view of earlier observations on the reversal of inhibition in *Streptococcus faecalis* — PGA bioassay systems by high levels of nicotinic acid and ascorbic acid (14). Coenzyme II ¹) also exhibited some activity in this respect, perhaps as the result of conversion to coenzyme I.

Summary.

1. A series of 1,2-dihydro-*s*-triazines has been studied in *Lactobacillus casei* # 7469 — riboflavin, *Lactobacillus fermenti* # 9338 — thiamine, and *Lactobacillus arabinosus* # 8014 — nicotinic acid bioassay systems. The compounds studied were relatively poor inhibitors in riboflavin and thiamine bioassay systems, inhibition in either case being reversed by high concentrations of the metabolic derivatives of pteroylglutamic acid, as well as by high con-

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¹) Product of the Sigma Chemical Company, St. Louis, Missouri.

centrations of riboflavin or thiamine in the respective bioassay systems.

2. Activity in *Lactobacillus arabinosus* ⋕ 8014 bioassay systems varied with substitution in the molecule, the 2,2-dimethyl-derivative with 3′, 4′-dichlorophenyl substitution exhibiting the most activity. Inhibition was reversed by nicotinic acid, nicotinamide, or coenzyme I, as well as by the metabolic derivatives of pteroylglutamic acid. Certain derivatives also interfered with the utilization of *para*-aminobenzoic acid in appropriate bioassay systems.

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CHEMICAL AND BIOLOGICAL STUDIES ON 1,2-DIHYDRO-*S*-TRIAZINES

IX. INHIBITION OF SYNTHESIS OF CITROVORUM FACTOR IN *LEUCONOSTOC CITROVORUM* # 8081 SYSTEMS [1]

by

G. E. FOLEY and **E. C. HALEY**

(Received March 30, 1955).

INTRODUCTION.

Several reports indicate that there is an intimate interrelationship between the metabolism of pteroylglutamic acid (PGA) and ascorbic acid. This relationship has been implicated directly or indirectly in hematapoiesis (42), megaloblastic anemia of infancy (26), the anemia accompanying scurvy (14), and the stimulation of growth in chicks (3). Other studies have indicated that PGA duplicates the effect of ascorbic acid in correcting abnormal tyrosine metabolism in scorbutic guinea pigs (36, 44) and PGA-deficient rats (32). Another aspect of this interrelationship concerns the synthesis of citrovorum factor (CF) [2]. The urinary excretion of CF has been shown to be roughly proportional to PGA intake in rats (28, 35) and in humans (1), while there is considerable evidence that ascorbic acid and other reducing agents are related to the conversion of PGA to CF, as has been demonstrated in rats, in liver enzyme systems (28), in humans suffering from scurvy (14) and in bacteria (6, 17).

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[1] Abstract published in J. Bact. **66**, 727, 1953; Part VIII: Antonie van Leeuwenhoek **21**, 397, 1955.

[2] We wish to express our appreciation to the Lederle Laboratories, Pearl River, New York, for a generous supply of this compound.

Since the observation that *Leuconostoc citrovorum* # 8081 [1]) (*Pediococcus* ?) (9) requires CF as an essential metabolite (33), it has been presumed that this microörganism is unable to synthesize CF from minimal concentrations of PGA. During the course of studies on the inhibitory activity of a series of 1,2-dihydro-*s*-triazines (11—13), it was observed that the addition of high levels of ascorbic acid to media containing otherwise ineffective concentrations of PGA supported near maximal growth of *Leuconostoc citrovorum* in the absence of preformed CF. Variations in the results obtained with modified media indicated that the response to such concentrations of PGA was determined by the concentration of reducing agents such as glutathione, *l*-cysteine, etc. in the medium in addition to (or instead of) ascorbic acid. The purpose of the present report is to describe the investigations which developed as the result of this observation. Media and methods of bioassay were as previously described (11—13).

Results.

The response of *Leuconostoc citrovorum* to PGA in the presence and absence of ascorbic acid is illustrated in Table 1. Concentrations of less than 5 gamma/ml of PGA did not support growth in the absence of ascorbic acid, while near maximal growth was obtained with 1–2 gamma/ml of PGA in the presence of high concentrations of ascorbic acid. Ascorbic acid had little effect on the ability of *Streptococcus faecalis* # 8043 [1]) to grow in the presence of sub-optimal concentrations of PGA or on the response of *Leuconostoc citrovorum* to CF, suggesting that the response of *Leuconostoc citrovorum* to PGA plus ascorbic acid was not due simply to increased utilization of sub-optimal concentrations of metabolites. However, the response of *Leuconostoc citrovorum* to increasing concentrations of ascorbic acid in the presence of various concentrations of PGA indicated a linear relationship between PGA and ascorbic acid at concentrations of the latter which yield maximal and near-maximal growth (Fig. 1).

The synthesis of CF or CF-like compounds by washed, buffered suspensions of *Leuconostoc citrovorum* from PGA in the presence of suitable concentrations of ascorbic acid has been demonstrated in experiments similar to those described by Bond (2) with *Lacto-*

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[1]) American Type Culture Collection number.

TABLE 1.

Response of *Leuconostoc citrovorum* # 8081 to PGA in presence and absence of ascorbic acid.

	Optical Density, 48 hrs.				
	Pteroylglutamic acid, gamma/ml				
Ascorbic Acid gamma/ml	10.0	5.0	2.0	1.0	0.5
5000	6.80	6.80	6.60	5.40	1.30
2500	6.90	6.80	5.40	3.90	0.60
1250	6.80	6.80	4.60	2.20	0.80
625	6.60	6.20	1.40	1.50	0.70
312	6.00	6.20	2.00	1.00	0.60
156	6.00	6.30	0.70	0.70	0.30
78	6.10	7.30	0.30	0.60	0.30
39	6.10	4.80	0.30	0.30	0.30
18	5.80	6.40	0.20	0.20	0.20
None	6.00	4.60	0.10	0.10	0.10

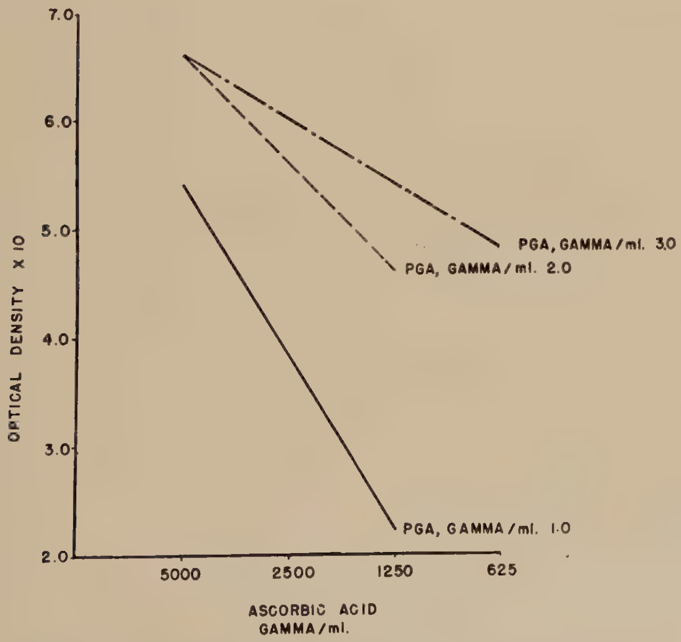

Fig. 1. Response of *Leuconostoc citrovorum* to increasing concentrations of PGA in the presence of ascorbic acid.

TABLE 2.

CF activity of filtrates of suspensions of *Leuconostoc citrovorum* # 8081 incubated with various metabolites.

Additions to Vessels, gamma/ml			CF Activity per 5 ml cell-free filtrate [1])
PGA	Ascorbic Acid	Sodium Formate	
2	0	0	0.5 units
2	5000	0	2.0–3.0 units
2	0	5000	0.55 ,,
2	5000	5000	5.0–7.0 ,,
0	5000	5000	0.0
2	5000	5000	0.0 [2])
2	5000	5000	0.0 [3])
0	0	0	0.0

[1]) As assayed with *Leuconostoc citrovorum.*

[2]) Cell suspension inactivated by boiling 10 minutes.

[3]) No cells to vessel.

bacillus casei. The yields of CF were low, from 2–3 units per 5 ml of cell-free filtrate, but were increased by the simultaneous addition of sodium formate to the system (Table 2). These values probably do not represent total yield, since no attempt was made to determine the CF content of the cells. Similar responses to ascorbic acid and sodium formate have been observed with other microorganisms (6, 17).

Ascorbic acid was inactive in the absence of PGA and in the presence of high concentrations of *para*-aminobenzoic acid. High concentrations (to 5.0 mg/ml) of sodium formate and/or *l*-serine neither replaced nor enhanced ascorbic acid or PGA activity, and similarly, did not enhance the utilization of CF by *Leuconostoc citrovorum.* The growth stimulatory activity of ascorbic acid was not due to its effect on the pH of the medium. Although its activity was not effected by autoclaving, either in pure solution or in the medium, solutions in N/1 NaOH were inactive. D-*iso*-ascorbic acid and vitamin B_{12} also were active, as were a number of other reducing agents (Table 3). As with ascorbic acid, there also was a linear relationship between the concentration of these latter compounds and extent of growth in the presence of a constant concentration of PGA (Fig. 2).

Vitamin B_{12} similarly duplicates the effects of reducing agents

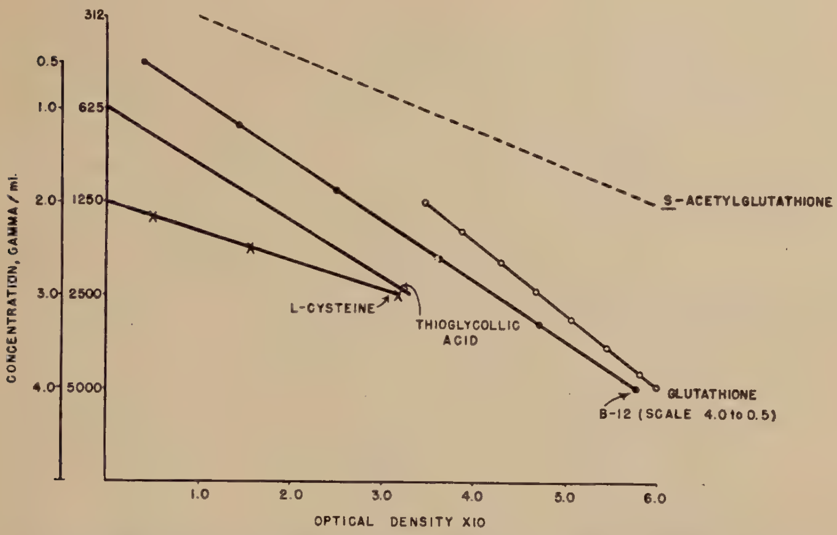

Fig. 2. Response of *Leuconostoc citrovorum* to increasing concentrations of reducing agents in presence of constant concentration of PGA.

in the nutrition of *Lactobacillus lactis* Dorner (16, 39), as does thymidine (24, 45). The effect of reducing agents on the growth of lactic acid bacteria (20, 21, 30, 38, 40, 41, 43) generally has been attributed to the protection of vitamin B_{12} during autoclaving in basal media. Welch and Wilson (43) reported that ascorbic acid was without effect in an amino acid medium used for the cultivation of *Lactobacillus leichmannii*, but that ascorbic acid and other reducing agents potentiated the inherent vitamin B_{12} activity of tryptic digested casein (31). It seems unlikely that such is the explanation of the present results, since the growth response of *Leuconostoc citrovorum* to CF or PGA and ascorbic acid in varying concentrations was the same in media containing acid hydrolyzed or tryptic digested casein. Similarly, the addition of high levels of tryptic digested casein to acid hydrolyzed casein media before or after autoclaving did not replace either PGA or the need for a reducing agent. Other experiments failed to demonstrate any evidence for an additive or synergystic effect between low concentrations of vitamin B_{12} and reducing agents, either when autoclaved separately or together in basal media, or added separately or together to sterile basal media. Poor response was obtained to combinations of PGA and reducing agents in the absence of purines in the basal

TABLE 3.

Effect of substances *) other than ascorbic acid on reponse of *Leuconostoc citrovorum* # 8081 to PGA.

Derivatives of Ascorbic Acid	
Active	Inactive
d-iso-Ascorbic Acid	Dehydro Ascorbic Acid
	Methyl Ascorbic Acid [1])
	Sodium Ascorbate
	Ferrous Ascorbate
Precursors of Ascorbic Acid	
	d-Sorbitol
	l-Sorbose
Metabolic End Products of Ascorbic Acid	
	Alloxan
	Dialuric Acid
	Alloxantin
Other Reducing Agents	
Glutathione	Reduced Glutathione
S-Acetyglutathione	Hydroquinone
l-Cysteine	Resorcinol
Thioglycollic Acid	*p*-Aminophenol
Sodium Thioglycollate (weak)	*p*-Phenylene Diamine
Vitamin B_{12} [3])	Methylglyoxal [2])
	Butylhydroxyanisole
	Alpha Tocopherol [3])
	l-Cystine
Miscellaneous	
	Glucuronic Acid
	Glucuronic Acid Lactone
	d-Gulose, *l*-Gulose
	Gulonic Lactone
	Kojic Acid
	Sodium Formate
	l-Serine
	Sorbitan Monooleate Complex
	Orotic Acid [4])

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*) Studied in concentrations of 5000–156 gamma/ml in presence of 2 gamma/ml of PGA unless otherwise noted.

[1]) This compound was supplied through the kindness of Dr B. S. Gould, Department of Biochemistry, Massachusetts Institute of Technology, Cambridge, Mass.

[2]) High concentrations (more than 0.5–1.0 mg/ml) inhibitory for *Leuconostoc citrovorum* in presence of citrovorum factor.

[3]) 4.0 gamma/ml maximum concentration studied.

[4]) Limited to 100 gamma/ml by insolubility in aqueous substrate.

TABLE 4.

Effect of dihydrotriazine hydrochlorides on response of *Leuconostoc citrovorum* # 8081 to PGA in presence of ascorbid acid.

Inhibitor gamma/ml	Optical Density, 48 hrs.	
	PGA 2.0 gamma/ml	PGA 2.0 gamma/ml Ascorbic acid 5000 gamma/ml
D-54 . HCl [1]		
0.80	0.00	0.00
0.60	0.00	0.00
0.40	0.00	0.00
0.20	0.00	0.00
0.10	0.00	0.00
0.05	0.00	2.87
0.025	0.00	4.90
0.0125	0.00	4.80
0.00625	0.00	5.50
None	0.00	5.80

[1] 4,6-diamino-1-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride. Two samples of this compound were used in these studies, one synthesized in these laboratories by Dr E. J. Modest and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

medium, as would be expected, in view of Sauberlich's observation (34) that unlike *Lactobacillus lactis*, vitamin B_{12}, PGA or even CF does not replace the purine requirement of *Leuconostoc citrovorum*.

The reversibility of phenyldihydrotriazine inhibition in *Streptococcus faecalis* — PGA bioassay systems by high levels of reducing agents (13, 27) or reduced (dihydro-) PGA (11–13, 27), suggested that reducing agents permit the reduction of PGA and the subsequent synthesis of CF by *Leuconostoc citrovorum*. The effect of the addition of phenyldihydrotriazines to the *Leuconostoc citrovorum* — PGA bioassay system containing a reducing agent is illustrated by the experiments summarized in Table 4. Concentrations of 0.1 gamma/ml or more of D–54 · HCl prevent the growth of *Leuconostoc citrovorum*. This concentration (0.1 gamma/ml) compares favorably with that required (0.28—0.3 gamma/ml = 50% inhibition) for the inhibition of excess concentrations of PGA in *Streptococcus faecalis*, and is in sharp contrast to those required

TABLE 5.
Response of *Leuconostoc citrovorum* ⋕ 8081 to pteroylglutamic acids in the presence and absence of ascorbic acid.

Metabolite	Gamma/ml	Optical Density, 48 hrs. Alone	Optical Density, 48 hrs. Ascorbic acid 5000–312 gamma/ml
Pteroylglutamic acid	10.0	6.30	7.00
	2.0	0.00	5.80
	1.0	0.00	4.90
	0.1	0.00	0.45
Dihydropteroylglutamic acid [1])	10.0	8.00	8.10
	1.0	8.50	8.00
	0.1	7.60	7.90
	0.01	3.10	7.90
	0.001	0.40	5.40
	0.0001	0.20	4.70
N^{10}-formylpteroyl-glutamic acid	10.0	1.30	6.70
	2.0	0.00	5.40
	1.0	0.00	1.60
	0.1	0.00	0.80

[1]) Presumably the 7,8-dihydro-derivative, prepared by hydrogenation of pteroylglutamic acid over platinum oxide in alkali according to O'Dell (29) by Dr E. J. Modest in these laboratories.

for the inhibition of CF in *Streptococcus faecalis* (11.6 gamma/ml = 50% inhibition) and *Leuconostoc citrovorum* (22 gamma/ml = 50% inhibition) bioassay systems. The addition of reduced PGA[1]) (29) to such inhibited systems resulted in reversal, indicating the biosynthesis of CF. Other studies further indicated that reduced PGA can be substituted for CF in *Leuconostoc citrovorum* bioassay systems, and that if reduced PGA is provided, the potentiating effect of ascorbic acid and other reducing agents disappears except at very low concentrations of metabolite (Table 5). Another derivative, N^{10}-formyl-PGA [1]) (15) which requires reduction if used as a precursor for CF (4, 10), is identical with PGA in its inability to support the growth of *Leuconostoc citrovorum* in this bioassay system at low concentrations in the absence of ascorbic acid, as well as its potentiation by high levels of reducing agent (Table 5).

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[1]) We wish to express our appreciation to the Lederle Laboratories, Pearl River, New York, for a generous supply of this compound.

Discussion.

The role of ascorbic acid in bacterial metabolism has been reviewed recently by Eddy and Ingram (8), and in those instances where a relationship has been established, the effect on redox potential appears to be its most significant function. Similarly, recent investigations indicate that the role of ascorbic acid in the oxidation of tyrosine (25) is to provide the proper redox potential, rather that that of a cofactor for an enzyme catalyzing an oxidative reaction, as had been suggested earlier (37). The present results on the participation of reducing agents in the biosynthesis of CF by *Leuconostoc citrovorum* coincide with those obtained in other biological systems (2, 6, 17, 28). Furthermore, because the present findings were not predicated upon the enzymatic liberation of CF, they agree with those of Hendlin, Koditschek and Soars (17), and do not support the view that this activity of ascorbic acid is the result of its participation in a CF-liberating system (18, 19). A similar example of a biosynthesis dependent upon reducing agent has been reported by Katznelson (23) with *Bacillus paraalvei*, which synthesizes its thiamine requirement only in the presence of suitable reducing agents.

The linear relationships between extent of growth and reducing agent in the presence of otherwise ineffective concentrations of PGA suggest that the reducing agent is intimately related to the conversion of PGA to CF. The diversity of reducing agents which exhibit activity, as well as the relatively high concentrations required, suggest that these relationships are the result of a physicochemical effect on redox potentials rather than direct participation in the reaction. The results reported in other bioassay systems (6, 17), particularly those of Broquist *et al.* (6) who demonstrated that a nitrogen atmosphere increases the biosynthesis of CF by *Streptococcus faecalis*, suggest a similar conclusion. The ability of reduced PGA to replace PGA and reducing agent indicates that the need for a reducing agent is associated with the reduction of PGA. The potentiating effect of reducing agents perhaps may be direct *via* activation of an enzyme system, or indirect *via* protection of a labile intermediate. since Broquist *et al.* (5) reported that tetrahydro-PGA which is oxygen-sensitive, also may be substituted for CF with *Leuconostoc citrovorum*.

The activity of vitamin B_{12} in this bioassay system is of particular interest. HENDLIN, KODITSCHEK and SOARS (17) were unable to demonstrate any effect with vitamin B_{12} on the conversion of PGA to CF by *Streptococcus faecalis* and *Lactobacillus lactis*. Although the former synthesizes, the latter requires preformed vitamin B_{12} (or a substitute), hence the lack of an effect suggested that vitamin B_{12} does not participate in the bacterial conversion of PGA to CF. On the other hand, studies by DIETRICH, NICHOL, MONSON and ELVEHJEM (7) suggested that vitamin B_{12} may enhance the conversion of PGA to CF in chicks. In the present studies, higher levels of vitamine B_{12} than those studied (17) replaced reducing agents. Earlier reports on the substitution of reducing agents for vitamin B_{12} (16, 30, 39, 41, 43) suggest that the activity of this metabolite may be at least in part due to an effect on biological redox potentials, and the present experiments suggest that vitamin B_{12} may be concerned with the conversion of PGA to CF. It is known that vitamin B_{12} is synthesized by *Leuconostoc citrovorum* (22); however, inability to synthesize this metabolite in sufficient quantity may be the defect which precludes the synthesis of CF in the absence of reducing agents. A similar interpretation also might apply to the relationship between vitamin B_{12} and the synthesis of desoxyribosides as suggested by JUKES, BROQUIST and STOKSTAD (22).

Summary.

The addition of high levels of ascorbic acid to media containing otherwise ineffective concentrations of pteroylglutamic acid supported near maximal growth of *Leuconostoc citrovorum* ♯ 8081 in the absence of preformed citrovorum factor. Studies with resting cell suspensions demonstrated the synthesis of citrovorum factor activity in such systems. Ascorbic acid could be replaced by *S*-acetylglutathione, glutathione, thioglycollic acid or *l*-cysteine. The activity of reducing agents of such diverse chemical structure suggests a physico-chemical effect rather than a specific metabolite response. The role of vitamin B_{12} in replacing reducing agents in this reaction is discussed.

Reduced (dihydro) pteroylglutamic acid could be substituted

for combinations of pteroylglutamic acid and reducing agents or preformed citrovorum factor, suggesting that the proper redox potential permits the reduction of pteroylglutamic acid and subsequent biosynthesis of citrovorum factor activity by *Leuconostoc citrovorum*. This biosynthesis was inhibited by the 1,2-dihydro-*s*-triazines, and inhibition could be reversed by reduced pteroylglutamic acid, as well as citrovorum factor.

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CHEMICAL AND BIOLOGICAL STUDIES ON 1,2-DIHYDRO-*S*-TRIAZINES

X. MECHANISM OF ACTION IN MICROBIOLOGICAL SYSTEMS [1]

by

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INTRODUCTION.

Studies previously reported on the microbiological activity of a series of 1,2-dihydro-*s*-triazines (6, 7, 10, 11, 12) have led to the formulation of a concept of the locus of inhibition by these compounds which, together with additional observations on the reversal of inhibition in microbiological systems, will be presented here. Media and methods were as described previously (6, 7, 10, 11, 12) unless otherwise noted.

It is now generally accepted that citrovorum factor (CF) is a biologically active derivative of pteroylglutamic acid (PGA) as postulated by SAUBERLICH (36) and is one of the forms or is closely related to the form in which PGA exhibits coenzyme activity. The chemical synthesis of CF from PGA involves at least two steps, formylation and reduction (2, 5, 27, 33, 34), and ascorbic acid is known to mediate the reductive step (27). There also is evidence suggesting that the biosynthesis of CF from PGA may be accomplished in a similar manner (1, 14, 30, 31, 36). NICHOL and WELCH (30) described the conversion of PGA to CF in liver bioassay systems and its enhancement by ascorbic acid. Other studies by these authors (31) further indicated that this biosynthesis also involves at least two steps, the first of which may be blocked by 4-aminopteroylglutamic acid [1] (4-APGA), while the second, which is mediated by ascorbic acid, is not affected by 4-APGA. N^{10}-formylpteroylglutamic acid [1] (N^{10}-formyl-PGA) (16), dihydropteroylglutamic acid [1] (reduced PGA) (26) and CF [1] (2, 5, 27, 33, 34, 36) are known to replace PGA and to reverse inhibition by the analogues of PGA in microbiological systems, suggesting that the reduced and formylated intermediates as well as CF may occur and be utilized in microbiological systems. Thus, it seemed reasonable to assume that the metabolism of PGA by the bacterial cell may proceed in a similar manner; and if PGA is concerned here with the synthesis of purines and other precursors in the formation of nucleic acids as it appears to be in other biological systems (43), then PGA must be formylated, reduced and used early in the course of cell growth, since nucleic acids increase and accumulate in the bacterial cell early in the lag phase prior to cell division (41). Consequently, experiments were undertaken to determine the time during the growth cycle in which the metabolism of PGA could be interrupted by the addition of inhibitor to growing cultures.

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[1] Part IX: Antonie van Leeuwenhoek **21**, 405, 1955.

TIME EXPERIMENTS.

These experiments with *Streptococcus faecalis* # 8043 [2] and *Lactobacillus casei* # 7469 [2] growing in the presence of PGA are summarized in Figures 1 and 2 respectively. The addition of D–54 . HCl [3] to *Streptococcus faecalis* after as little as 4 hours incubation had little effect on the growth curve, while the effective interval

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[1] We wish to express our appreciation to the Lederle Laboratories, Pearl River, New York, for generous samples of these compounds. Another sample of dihydropteroylglutamic acid, synthesized by E. J. M. according to O'DELL, *et al.* (26), by hydrogenation of PGA over PtO_2 in alkali, also was used in these studies. It has been proposed (26) by analogy with model compounds that dihydro-PGA prepared in this manner is the 7,8-dihydroderivative. However, the exact location of the two hydrogen atoms has not been unequivocally determined and other structures can be written for dihydro-PGA with reduction in the pyrazine ring. If dihydro-PGA should exist in more than one form, there might be differences in biological properties.

[2] American Type Culture Collection numbers.

[3] Two samples of this compound were used in these studies, one synthesized by E. J. M. in these laboratories, and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England. See Table 5 for structure of D-54 . HCl and D-69 . HCl.

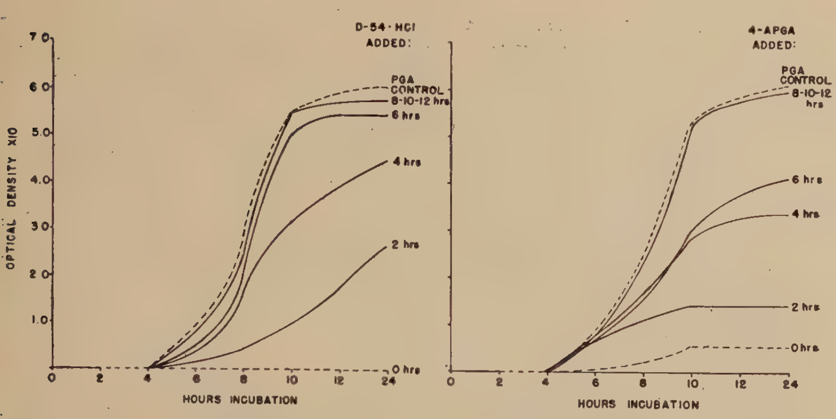

Fig. 1. Effect of addition of inhibitors to *Streptococcus faecalis* # 8043 growing in presence of 0.01 gamma/ml of PGA. Inhibitor concentrations: D–54 . HCl, 0.3 gamma/ml; 4–APGA, 0.1 gamma/ml.

for 4-APGA was *ca.* 6 hours. The addition of D–69 . HCl to *Lactobacillus casei* after 6 hours was without significant effect, while the effective interval for 4-APGA again was longer, *ca* 14 hours. The reverse experiments, in which metabolites were added after increasing intervals to cultures incubated in the presence of inhibitory concentrations of either phenyldihydrotriazine or 4-APGA, indicated that the addition of CF to either bioassay system even after

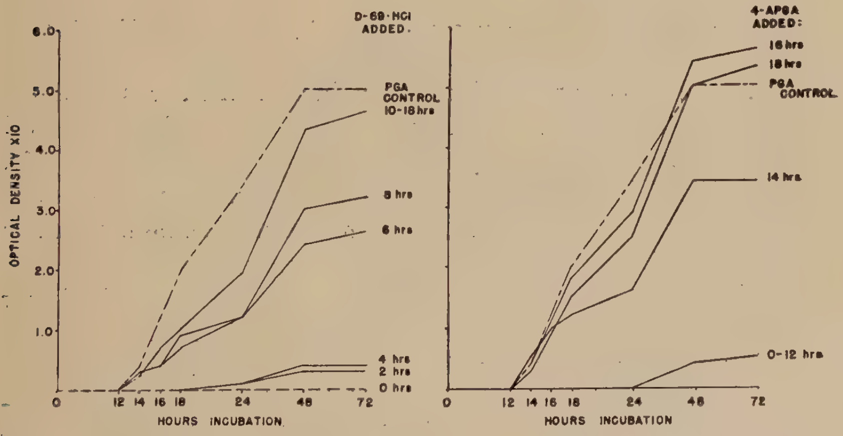

Fig. 2. Effect of addition of inhibitors to *Lactobacillus casei* # 7469 growing in presence of 0.001 gamma/ml of PGA. Inhibitor concentrations: D–69 . HCl, 15 gamma/ml; 4-APGA, 0.1 gamma/ml.

12–18 hours incubation resulted in the initiation of growth. The addition of PGA to such inhibited *Streptococcus faecalis* cultures had no effect, while *Lactobacillus casei* could be reversed competitively by PGA over the competitive range of the inhibitor (10). Thus, there appears to be a definitive time in the development of the bacterial culture after which the addition of these inhibitors has little effect. It is of interest to note that the results of these studies are similar to those obtained in mammals with respect to the temporal relationship of metabolites to reversal of the analogues of PGA (20).

REVERSAL OF INHIBITION.

Previous studies indicated that the phenyldihydrotriazines may be reversed by reduced PGA, CF, thymine or thymidine in appropriate bioassay systems (6, 7, 10, 11, 12). N^{10}-formyl-PGA also reversed low concentrations of inhibitor, but differed from reduced PGA in that it is relatively ineffective versus high concentrations (9). These results, in contrast to the irreversibility of inhibition by excess PGA, suggested that the locus of inhibition was at the reductive step in the conversion of PGA to CF, a hypothesis which was supported early in the course of these studies by the observation that high concentrations of ascorbic acid partially reversed inhibition in *Streptococcus faecalis* — PGA bioassay systems (28). Further investigation indicated that inhibition also may be reversed by other reducing agents (12) (Table 1), while studies with *Lactobacillus arabinosus* ♯ 8014 — nicotinic acid bioassay systems indicated that the dihydrotriazines were not simple antagonists of PGA, since coenzyme I ¹) reversed inhibition in this bioassay system (11) (Table 1). Similarly, coenzyme I partially reversed inhibition in *Streptococcus faecalis* bioassay systems (Table 1). Although the maximum concentration of inhibitor which was reversible with reducing agents in *Lactobacillus arabinosus* bioassay systems was limited (5–10 gamma/ml), lesser concentrations of nicotinic acid or coenzyme I reversed 2 or 3 times these inhibitor concentrations. These differences suggested that the effect of reducing agents was largely physico-chemical (12), while reversal by coenzyme I or its precursors was the result of coenzyme replacement, titration versus phenyldihydrotriazine in *Lactobacillus arabinosus* bioassay systems (Table 2) indicating that coenzyme I

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¹) Product of the Sigma Chemical Company, St. Louis, Missouri.

TABLE 1.

Comparison of reversibility of dihydrotriazine hydrochloride inhibition in different bioassay systems.

Additions to Basic Assay System	Concentration gamma/ml	Optical Density		
		Streptococcus [1] *faecalis* # 8043	*Lactobacillus* [2] *casei* # 7469	*Lactobacillus* [3] *arabinosus* # 8014
Ascorbic Acid	10,000–5,000	4.20	0.00	4.60
Glutathione	2,500	3.80	0.00	4.00
Thioglycollic Acid	2,500	1.20	0.00	[4]
Cysteine	2,500	3.70	0.00	4.00
Vitamin B_{12}	100	0.80	0.00	[4]
Coenzyme I	100	2.00	0.00	5.00 [5]
Nicotinic Acid	100	2.50	0.00	5.00
Nicotinamide	200	0.00	0.00	5.40
None		0.00	0.00	0.00
None — Assay Controls		4.20	4.80	5.00
Medium, no metabolite		0.00	0.00	0.00

[1] Basic systems contained PGA, 0.01 gamma/ml, D-54 . HCl, 1.0–0.5 gamma/ml. Read after 24 hours incubation.

[2] Basic systems contained PGA, 0.001 gamma/ml, D-54 . HCl, 20–10 gamma/ml. Read after 48 hours incubation.

[3] Basic systems contained nicotinic acid, 0.01 gamma/ml, D-54 . HCl, 10–5 gamma/ml. Read after 48 hours incubation.

[4] Results equivocal.

[5] Coenzyme II also partially reverses, perhaps via conversion to coenzyme I.

TABLE 2.

Inhibition of coenzyme I by D-54 . HCl in *Lactobacillus arabinosus* # 8014 bioassay systems.

D-54 . HCl gamma/ml	Optical Density, 48 hrs.			
	Coenzyme I		Nicotinic Acid	
	Gamma/ml			
	10.0	1.0	10.0	1.0
1000	0.00	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00
100	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00
1.0	3.80	3.60	0.10	0.10
0.1	4.80	5.00	3.70	4.00
None	7.70	7.00	7.00	6.80
Medium, no metabolite	0.00	0.00	0.00	0.00

Coenzyme I appears to be about 10 times as active as nicotinic acid by weight, or 50–60 times as active on a molar basis.

was somewhat more effective than nicotinic acid in reversing inhibition. Similar observations also were made with *Streptococus faecalis*, but although the minimal effective concentrations were considerably less than those of the effective reducing agents, complete reversal of inhibition could not be obtained with nicotinic acid or coenzyme I, suggesting that neither metabolite was utilized efficiently under these experimental conditions.

The observation that *Leuconostoc citrovorum* # 8081 synthesizes CF activity from otherwise ineffective concentrations of PGA in the presence of suitable concentrations of reducing agent, and that reduced PGA may be substituted for either PGA and reducing agent or preformed CF (8, 12), provided additional data on mechanism of action. Phenyldihydrotriazine inhibited the growth of *Leuconostoc citrovorum* in the presence of excess concentrations of PGA (Table 3); however, the addition of reducing agent reversed this inhibition over a 10-fold range of concentration of inhibitor. Vitamin B_{12} also reversed low concentrations of inhibitor as illustrated in Table 3, while high concentrations of nicotinic acid effected 20–30% reversal, but coenzyme I and II [1]) even in concentrations of 1 mg/ml were ineffective.

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[1]) Product of the Chemical Company, St. Louis, Missouri.

TABLE 3.

Reversal of dihydrotriazine hydrochloride inhibition of *Leuconostoc citrovorum* # 8081 — PGA systems by ascorbic acid and vitamin B_{12}.

Inhibitor, gamma/ml	Optical Density, 48 hrs.		
	PGA, 5.0 gamma/ml	PGA, 5.0 gamma/ml, Ascorbic Acid, 5000 gamma/ml	PGA, 5.0 gamma/ml, Vitamin B_{12}, 4.0 gamma/ml
D-54 . HCl			
0.05	0.00	2.87	0.00
0.025	0.00	4.90	0.00
0.0125	0.00	4.80	0.00
0.00625	0.00	5.00	6.50
None	6.40	6.80	5.50
Media, no metabolite	0.00	0.00	0.00

SYNTHESIS OF COENZYME I BY *Str. faecalis* and *L. citrovorum.*

Although microörganisms which require nicotinic acid usually utilize coenzyme I as well, there are instances in which the coenzyme is less effective than its precursors (24, 35). As indicated in Table 4, nicotinic acid or one of its derivatives is essential for the growth of *Leuconostoc citrovorum*, nicotinamide being more effective than

TABLE 4.

Utilization of nicotinic acid and certain of its derivatives by *Leuconostoc citrovorum* # 8081.

Metabolite	Gamma/ml	Optical Density, 48 hrs.	
		CF, gamma/ml	
		0.001	None
Nicotinic acid	1.0	7.00	0.00
	0.1	6.80	0.00
	0.01	3.60	0.00
	0.001	0.07	0.00
Nicotinamide	0.1	6.80	0.00
	0.01	6.80	0.00
	0.001	4.00	0.00
Coenzyme I	10.0	5.80	0.00
	1.0	5.80	0.00
	0.1	0.80	0.00

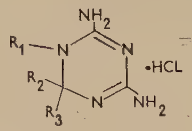
either nicotinic acid or coenzyme I. Experiments similar to those described by Koser and Kasai with *Leuconostoc dextranicum* (24) indicated that both *Leuconostoc citrovorum* and *Streptococcus faecalis* synthesize a factor from nicotinic acid which satisfies the coenzyme I requirement of *Hemophilus parainfluenzae* ⌗ 4101 as determined by the bioassay of appropriate culture filtrates according to the method described by Gingrich and Schlenk (15). The coenzyme I activity of such filtrates was low, *ca* 0.001 gamma/ml; however, they supported the growth of *Leuconostoc citrovorum* in the absence of nicotinic acid or its derivatives. Since the coenzyme I activity of these filtrates represents but a fraction of the concentration of exogenous coenzyme I required for growth (Table 4), these results suggest that the failure of exogenous coenzyme I to reverse inhibition in *Leuconostoc citrovorum*, as well as its inefficiency in *Streptococcus faecalis* bioassay systems, may be due to the relative unavailability of exogenous coenzyme I to the cell, perhaps because of differences in diffusibility in these species as compared to other bacterial cells, *e.g.*, *Lactobacillus arabinosus*.

INHIBITION OF COENZYME I ACTIVITY IN OTHER MICROBIOLOGICAL SYSTEMS.

Studies in other bacterial systems which involve the synthesis and utilization of coenzyme I but do not respond to preformed PGA or its derivatives also indicated that coenzyme I is inhibited by this series of 1,2-dihydro-*s*-triazines. *Corynebacterium diphtheriae* utilizes nicotinic acid (29, 46), while *Hemophilus parainfluenzae* requires but does not synthesize coenzyme I (25). The results of the study of this series of compounds versus „wild" strains of *Corynebacterium diphtheriae* in the medium described by Wadsworth (44) supplemented by minimal concentrations of yeast extract, and *Hemophilus parainfluenzae* in the medium described by Gingrich and Schlenk (15) are summarized in Table 5. In general, the same variations in microbiological activity resulting from structural alterations in the molecule were observed as described previously in bioassay systems (6, 7, 10, 11, 12). The inhibition of *Corynebacterium diphtheriae* was essentially noncompetitive and was reversed by nicotinic acid or nicotinamide as well as by coenzyme I. Nicotinic acid and nicotinamide were inhibited with equal effectiveness, *e.g.*, inhibition indices of 5.4 were obtained with D-20 . HCl versus 10 gamma/ml of either

TABLE 5.

Activity of phenyldihydrotriazine hydrochlorides in *Corynebacterium diphtheriae* var. *mitis* [1] and *Hemophilus parainfluenzae* systems.

Compound	Structure 			50% Inhibition Index	
	R_1	R_2	R^3	*C. diphtheriae* Nicotinic acid, 0.1 gamma/ml	*H. parainfluenzae* Coenzyme I, 0.001 gamma/ml
D–23	phenyl	methyl	methyl	1,500	31,000
D–20	4′-chlorophenyl	methyl	methyl	180	30,000
D–69	3′-chlorophenyl	methyl	methyl	960	7,500
D–68	2′-chlorophenyl	methyl	methyl	more than 10,000	500,000
D–40	4′-chlorophenyl	pentamethylene		more than 10,000	400,000
D–66	phenyl	methyl	ethyl	1,750	42,000
D–45	4′-chlorophenyl	H	methyl	1,000	60,000
D–48	4′-chlorophenyl	H	*n*-propyl	900	40,000
D–84	4′-chlorophenyl	H	*n*-hexyl	270	6,000
D–54	3′, 4′-dichlorophenyl	methyl	methyl	160	3,000
D–63	3′, 4′-dichlorophenyl	H	ethyl	600	60,000
D–95	3′, 4′-dichlorophenyl	H	*n*-hexyl	30	1,000

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1) "Wild" strains. Similar results were obtained with *gravis* and *intermedius* types; however, their character of growth did not permit accurate determination of optical density.

precursor, while coenzyme I was inhibited somewhat more effectively; an inhibition index of 0.57 was obtained with the same compound versus 100 gamma/ml of coenzyme I [1]) (9). The inhibition of the utilization of coenzyme I by *Hemophilus parainfluenzae* was more competitive, and could be reversed competitively by excess coenzyme I (9). High concentrations of reducing agents exhibited little effect, while vitamin B_{12} or PGA and its derivatives had no effect on inhibition in either bacterial system. The available data, particularly those obtained with *Hemophilus parainfluenzae,* indicate that the phenyldihydrotriazines interfere with the utilization rather than the biosynthesis of coenzyme I; however, since nicotinamide riboside was not available for study, final conclusion must await further investigation.

COMPARISON OF PHENYLDIHYDROTRIAZINES AND ANALOGUES OF PGA.

The fundamental difference between phenyldihydrotriazine inhibition and that of analogues of PGA (6, 7) is further emphasized by the failure of reducing agents or coenzyme I and its precursors to reverse analogue inhibition. Similarly, these agents failed to reverse phenyldihydrotriazine inhibition in *Lactobacillus casei* bioassay systems, again indicating that the mechanism of action in this bioassay system differs from that in *Streptococcus faecalis* — PGA bioassay systems. The similarity of the inhibition indices obtained versus either PGA or CF with *Lactobacillus casei* (10) as contrasted to the marked increase in the inhibition indices when CF was substituted for PGA in *Streptococcus faecalis* bioassay systems (7), indicates that the mechanism of action versus *Lactobacillus casei* may be primarily the result of interference with the utilization rather than the biosynthesis of CF.

It has been reported that 4-amino-5-imidazolecarboxamide accumulates in bacterial cultures inhibited by the sulfonamides (40,42) or 4-APGA (48). More recent evidence indicates that this substance accumulates as the ribotide (18) and that it is very likely a normal metabolite. It has been considered to be a likely purine precursor (17, 38, 39) and more recently, a precursor of inosinic acid (18,47). If the normal utilization of this intermediate requires the participation of CF as postulated by BUCHANAN and SCHULMAN (4) and supported by GREENBERG (19), then phenyldihydrotriazine inhibition of microbiological systems utilizing CF also may result in accumulation of this compound. Similar speculation is permissible with respect to the 2,4-diamino-pyrimidines (21, 22), which are remarkably similar in steric configuration and biological action to the phenyldihydrotriazines; however, final conclusions must await further investigation.

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[1]) This difference in inhibition index is less pronounced on a molecular basis than on a weight basis. The molar inhibition indices against nicotinic acid (or nicotinamide) and coenzyme I are, respectively, 2.30 and 1.31. These calculations indicate that one or two molecules of phenyldihydrotriazine effectively antagonize one molecule of coenzyme I.

DISCUSSION.

These concepts of the mechanism of action of the phenyldihydrotriazines are summarized in Fig. 3. The two pathways from PGA to CF outlined in Fig. 3 may both occur in microbiological systems, as indicated by the utilization of synthetically prepared forms of reduced or formylated PGA directly in these systems, as well as their ability to reverse phenyldihydrotriazine and PGA analogue inhibition. The temporal relationships between inhibitor and

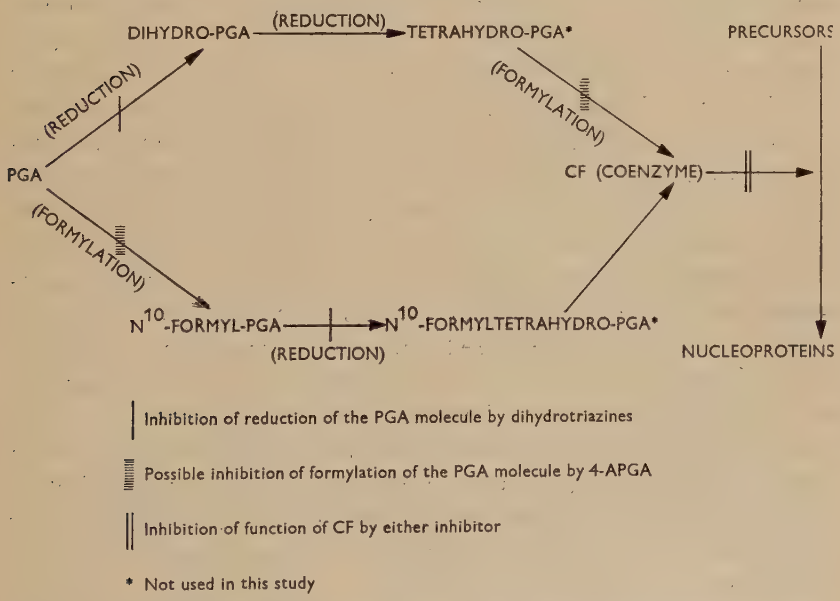
DIHYDRO-PGA
(REDUCTION)
TETRAHYDRO-PGA*
PRECURSORS
(REDUCTION)
(FORMYLATION)
PGA
CF (COENZYME)
(FORMYLATION)
N10-FORMYL-PGA
N10-FORMYLTETRAHYDRO-PGA*
(REDUCTION)
NUCLEOPROTEINS
Inhibition of reduction of the PGA molecule by dihydrotriazines
Possible inhibition of formylation of the PGA molecule by 4-APGA
Inhibition of function of CF by either inhibitor
* Not used in this study

Fig. 3. Postulated mechanism of action of 1,2-dihydro-s-triazines (and folic acid analogues) in microbiological systems.

metabolite with respect to the interval in the growth cycle in which inhibition can be effected indicate that the primary metabolic block occurs early in the lag phase coincident with the accumulation of nucleoprotein preparatory to cell division. The data derived from inhibition analyses (7, 11, 12) all indicate that the principal locus of activity of the phenyldihydrotriazines in microbiological systems can be placed at the reductive sequence in the biosynthesis of CF from PGA. The studies which illustrate the inhibition of CF by increased concentrations of inhibitor in *Streptococcus faecalis* and *Leuconostoc citrovorum* (7), and of either PGA or CF in *Lactobacillus casei* bioassay systems (10), suggest that like the analogues of PGA (3, 13, 37), these compounds do not exhibit a single mechanism action in all biological systems, but in addition to the efficient inhibition of the biosynthesis of CF, they also may interfere less effectively with the utilization of CF by the cell. There is some evidence that the phenyldihydrotriazines also may interfere with the utilization of PABA (11), in that high levels of PABA will partially reverse inhibition of *Lactobacillus arabinosus* — nicotinic acid systems. Reference to the locus of activity of the analogues of PGA is necessarily incomplete (Fig. 3), since precise data are not available. Inhibition by 4-APGA (and other analogues) has been located tentatively at the formylation step as a logical inference derived from consideration of the data presented by Nichol and Welch (30, 31) on the biosynthesis of CF by liver slices, together with the differences which the phenyldihydrotriazines and the analogues of PGA exhibited in microbiological systems. However, there is no direct evidence that PGA analogues inhibit the formylation of the PGA molecule.

The reversal of phenyldihydrotriazine inhibition by high concentrations of reducing agents appears to be a physico-chemical phenomenon associated with redox potential, as previously observed (12). Waksman *et al.* (45) recently reported that the mechanism of action of a new antibiotic "fradicin" was associated with biological redox and similarly, was reversed by suitable reducing agents. In the present instance, it is of interest to note that the effective reducing agents are associated with redox potentials suitable for the activity of enzyme systems concerned with the transfer of hydrogen.

The activity of coenzyme I and its precursors in reversing phenyldihydrotriazine inhibition indicates the participation of coenzyme I—dependent enzyme systems in the reduction of PGA to dihydro-PGA in microbiological systems, in accord with conclusions reached independently by KISLIUK and SAKAMI (23), GREENBERG (19), and NICHOL (32). KISLIUK and SAKAMI report that in pigeon liver systems coenzyme I (DPN) mediates this reduction, while GREENBERG, also using pigeon liver systems, concludes that DPN functions in the reduction of dihydro-PGA to tetrahydro-PGA. NICHOL has found that DPN is required for the conversion of PGA to CF in cell-free extracts of a PGA analogue-resistant strain of *Streptococcus faecalis*. Further investigation should reveal whether a coenzyme I-linked system is specifically required for the general biological reduction of PGA.

Inhibition of a coenzyme I-mediated reduction of PGA by the phenyldihydrotriazines indicates that these compounds function as coenzyme I antagonists rather than as antagonists of PGA *per se*; however it is possible that they may specifically inhibit this particular reduction. There are at least three different mechanisms which may be postulated as explanation of their activity: (1) inhibition of the synthesis of coenzyme I; (2) displacement of coenzyme I by the inhibitor on an apoenzyme; and (3) blocking of a reactive group essential to biological activity. Inactivation of a reactive group which is essential to biological activity but is not concerned with binding the substrate appears to be a logical explanation of the activity of these compounds in microbiological systems, since it is compatible with the inability of excess substrate (PGA) and the ability of excess enzyme (coenzyme I or its precursors) to reverse inhibition. The activity of reducing agents also could be explained by such a mechanism, in that proper redox potential may protect the reactive group. The role of vitamin B_{12} in the reversal of inhibition (12) might be similar.

Summary.

The data derived from analysis of 1,2-dihydro-*s*-triazine inhibition in a number of microbiological systems indicates that the primary mechanism of action of these compounds is interference with the reduction of pteroylglutamic acid in the biosynthesis of citrovorum factor, while higher concentrations also may interfere with the utilization of citrovorum factor in certain microbiological systems. There also is some evidence that the phenyldihydrotriazines interfere with the utilization of *p*-aminobenzoic acid.

The microbiological reduction of pteroylglutamic acid in the biosynthesis of citrovorum factor appears to involve a coenzyme I-dependent system, since coenzyme I or its precursors effectively reverse phenyldihydrotriazine inhibition in appropriate bioassay systems. Certain biological reducing agents or vitamin B_{12} also reverse inhibition, but are less efficient than coenzyme I in this respect. Interference with the utilization of coenzyme I and its precursors has been demonstrated with "wild" strains of *Corynebacterium diphtheriae*, while inhibition of coenzyme I also could be shown with *Hemophilus parainfluenzae* # 4101. The microbiological activity of the various derivatives of the series in these systems varied with structural alterations in the molecule, as observed previously with other bioassay systems.

The failure of coenzyme I, its precursors, certain biological reducing agents and vitamin B_{12} to effect the reversal of inhibition in *Lactobacillus casei* # 7469 bioassay systems suggests that the metabolism of pteroylglutamic acid by this microörganism differs in some respects from that observed in *Streptococcus faecalis* # 8043. Similarly, the failure of these agents to reverse inhibition induced by the analogues of pteroylglutamic acid provides further evidence of the fundamental difference in the mechanism of action between this class of compounds and the phenyldihydrotriazines. The possibility is considered that the analogues of pteroylglutamic acid interfere with formylation rather than the reduction of pteroylglutamic acid in the biosynthesis of citrovorum factor.

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SOME OBSERVATIONS ON THE LIPOLYTIC ACTIVITY OF MICRO-ORGANISMS AND A NEW METHOD FOR ITS DETECTION

by

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1. Introduction.

In the course of time several methods for the establishment of the lipolytic activity of micro-organisms have been described. In many instances it is of much importance to give a definite answer to the question whether a certain micro-organism has the ability to hydrolyse fats. This holds particularly in cases in which spoilage of food stuffs is apparently accompanied by decomposition of fatty components, leading to the phenomenon of rancidity. The deterioration of oil-water emulsions, like butter and margarine, offers well-known examples in this respect.

In such cases it is usually not difficult to isolate several microbial species from the aqueous phase, and then the question arises, if all these organisms are able to attack the oil, or that one or more are just profiting from the products of the lipolytic activity of some other species.

The better known methods for the detection of lipolytic activity can be grouped as follows:

1. The plate method of Eykman (1901) which still is widely in use in the Netherlands.
2. The oil-emulsion plate method introduced by Turner (1929), in which use is made of a dye as the indicator of lipolysis.
3. The tributyrin-emulsion plate of Anderson (1934).

These methods shall now be briefly discussed.

ad. 1. In the plate method as designed by Eykman the bottom of a Petri dish is covered with a very thin, translucent layer of tallow, on top of which a normal nutrient agar is poured. The plate is then inoculated in the usual way. Colonies under which after one or more days a white opaque spot in the tallow layer is formed are considered to be lipolytic. The change in appearance of the tallow is apparently due to the formation of insoluble calcium or magnesium soaps from the free fatty acids produced by the lipolysis.

It is evident that EYKMAN's method is based on the assumption that all lipolytic organisms will under the conditions of the experiment excrete a lipolytic enzyme, a lipase, into the medium surrounding the colony. Now it seems open to doubt whether this assumption will always hold. It could very well be that an organism yielding a negative result on an EYKMAN plate would nevertheless be able to attack fat, if a direct contact between the cells and a fat layer had been realized, as will apply to micro-organisms present in the aqueous phase of products such as butter and margarine. One could easily imagine that certain organisms, although endowed with potential lipolytic activity, abstain from excreting a lipase which may never meet a molecule of fat. This possibility is, moreover, considerably strengthened by the recent experiences concerning "induced enzyme synthesis" (MONOD and COHN, 1952). In several cases the formation of some hydrolytic enzyme has been proved to be strictly bound to the action of some more or less specific inducer on the cells.

A critical examination of the results of the EYKMAN method seemed, therefore, desirable.

ad. 2. In 1929 TURNER published his oil emulsion plate method in which he applied nile blue sulphate as an indicator for fat hydrolysis. A small quantity of a sterilized emulsion of fat or fatty oil in an 1.0% aqueous gum solution is thoroughly mixed with sterile melted nutrient agar to which 0.01% of nile blue sulphate had been added before sterilization. The mixture is then poured into a Petri dish, and the organism to be tested is streaked on the plate. In the vicinity of lipolytic colonies the oil droplets which initially were stained faintly red, take a deep blue colour.

Already TURNER himself reports that nile blue sulphate has a toxic effect on certain strains of micrococci and staphylococci, and this drawback of the method has been emphasized by later investigators. Several ways to avoid this complication have been proposed. LONG and HAMMER (1937) do not dissolve the dye in the medium, but flood the plate with an aqueous solution of the dye after incubation, as had already been suggested by TURNER. KNAYSI (1941, 1942) replaces nile blue by neutral red, dissolving this indicator in the fat instead of in the medium. STARR (1941, 1942) recommends spirit blue, and JONES and RICHARDS (1952) prefer victoria blue or night blue as an indicator.

Although there is no doubt that the later proposed dyes have a slighter toxic effect than nile blue, they are neither quite harmless to all types of bacteria. The same may hold for certain emulgators used by various authors for obtaining a satisfactory and stable oil emulsion. Moreover, it does not seem excluded that either the dyes or the emulgators inhibit lipase action; it is for instance certain that gum arabic markedly retards the decomposition of simple esters, such as methyl butyrate, by pancreatic lipase. Another drawback of the method is that in the case of weakly lipolytic organisms the colour change of the oil droplets can only difficultly be observed by the naked eye. If, however, recourse is had to the microscope the stain can as well be omitted, since fat hydrolysis manifests itself immediately in a change of the appearance of the oil drops.

Finally it will be clear that also with the plate emulsion method the result is judged in oil drops which are not in direct contact with the bacteria. In other words a positive result is only obtained, if the organism excretes a lipase into the medium.

ad. 3. The tributyrin agar method offers the advantage that on a plate containing an emulsion of the ester lipolytic organisms develop a clear zone around the colonies, since the products of the lipolysis are soluble in the aqueous medium. No indicator with possibly toxic properties is required. However, the method is not in general use, because it has been established with certainty that several micro-organisms which attack tributyrin are unable to bring about a hydrolysis of true fats (LONG and HAMMER, 1937).

In view of the above discussion, it will be clear that a satisfactory test method for lipolytic activity should answer the following requirements: *a.* direct contact between the organism and the oil phase, *b.* the use of indicators and emulgators should be avoided, *c.* a true fat should be used as substrate of the lipolysis.

2. NEW METHOD FOR THE DETECTION OF LIPOLYTIC ACTIVITY.

In the foregoing the desirability has been stressed to develop a method in which a direct contact between the cells of the organisms to be tested and the fatty oil is ensured, preferably already during the growth phase (Monod and Cohn, 1952).

In this connexion our attention was drawn to a publication of Webley (1953) who studied the growth of micro-organisms at the expense of liquid hydrocarbons in micro-cultures. For this purpose Webley applied a spray with the aid of which he deposited on a coverglass small droplets of a suspension of the organism in the hydrocarbon to be tested. The coverglass was then placed on a well slide, of which the well was filled with a mineral nutrient liquid. In droplets which contained one or only a few cells the growth of the organism could easily be observed under the microscope. As Webley himself suggests the method can also be applied to fatty oils instead of hydrocarbons. Nevertheless, it seemed to us that this method was not recommendable as a routine test. On the other hand the idea of using a sterile spray for obtaining a fine division of the oil was attractive, and this made us decide to spray finely divided oil droplets on a suitable agar plate medium, immediately after streaking the organism to be tested on the plate.

It could be expected — as preliminary experiments had shown — that an attack of the oil by the organism to be tested would manifest itself directly in the microscopic appearance of the droplets.

For the spraying of the oil use was made of a slightly modified form of the nebulizer described by Kluyver and Visser (1950). Fig. 1 gives a picture of the apparatus in question which was made by the glass-blower of this laboratory, Mr P. Wentink, who also was responsible for the prototype.

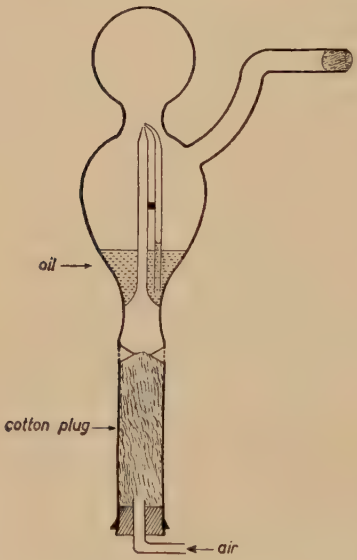

Fig. 1. Nebulizer.

After the nebulizer had been filled with olive oil and provided with cotton plugs, it was submitted to sterilization in the autoclave at 120°C. With the aid of the nebulizer oil droplets were sprayed on a previously inoculated agar medium from a distance of a few cm. It was found that this procedure only very seldom led to a contamination of the culture. It is recommendable to examine the plate under the microscope from the bottom side in order to make sure that an adequate density and distribution of the droplets has been obtained. The average diameter of the fairly uniform droplets on the plate should be in the neighbourhood of 30 μ.

It was found, that if the organism submitted to the test had the ability to bring about lipolysis the appearance of the droplets either in contact with, or situated in the immediate vicinity of the colonies, indeed showed an unmistakable change; the transparent droplets became irregular in shape, granular and opaque. Using a microscope with low magnification, and examining from the bottom side of the dish, this change can easily be established.

Fig. 2 and Fig. 3 show characteristic examples of the aspect of the droplets in cultures of a lipolytic and of a non-lipolytic bacterium respectively.

In most cases the change in the droplets already became visible within 24 hours: exceptionally organisms were encountered which showed lipolytic activity only after 3 to 5 days. We never found an organism which revealed lipolytic activity only after more than 5 days, although the plates were examined over a period of 20 or even 30 days.

As with the Eykman method the observed change in the droplets

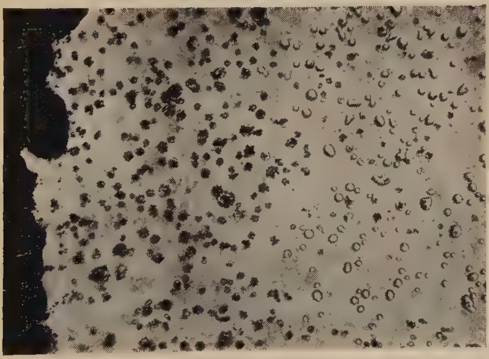

Fig. 2. Colony of a lipolytic organism (a *Sarcina*) on a plate sprayed with olive oil. From left to right: colony, zone of hydrolyzed oil droplets, unchanged oil droplets (30 ×).

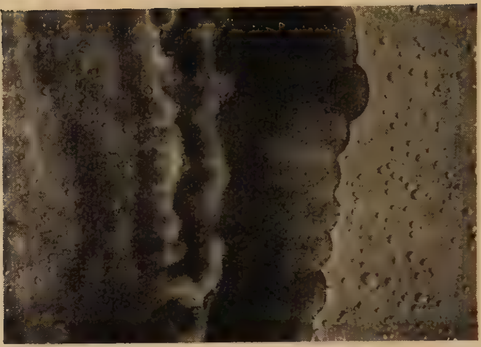

Fig. 3. Colony of a non-lipolytic organism (a *Micrococcus*) on a plate sprayed with olive oil. All droplets, whether in direct contact with the colony or not, have remained unchanged (30 ×).

must be due to the formation of insoluble soaps, caused by the presence of Ca and Mg ions in the medium. Oleic acid, sprayed upon a sterile peptone agar plate, changed within a few minutes from transparency to the appearance which is characteristic for the attacked oil droplets. On the other hand olive oil droplets did not change in appearance during a week in media of pH 8—10, so that this change is not due to an interaction of the oil with some constituent of the medium.

3. COMPARISON OF THE EYKMAN METHOD WITH THE SPRAY TEST.

It seemed desirable to compare the results of the new method with those obtained with the EYKMAN method. In the first place we wished to make sure that in all cases, in which the EYKMAN test yielded a positive result, also the spray method would give the same outcome. Moreover, as set forth in the Introduction, it did not seem excluded that certain strains giving a negative EYKMAN test would behave differently in the spray test, because of the direct contact between bacteria and oil. Finally it seemed of importance to give attention to the time necessary for the observation of a positive result in the two cases. A number of bacterial strains, amongst which several were known to be active fat splitters, was subjected to both tests. In all cases a plain peptone agar was used as nutrient medium. Table 1 gives a survey of the results obtained.

TABLE 1.

Lipolytic activity of various bacterial species.

Bacterial species	EYKMAN test	Spray test
Micrococcus albus 1	—	—
,, ,, 2	+ (5) cf. text	—
,, ,, 3	—	—
,, ,, 4	—	—
,, ,, 5	+ (3 à 6)	+ (1)
Micrococcus casei 1	—	—
,, ,, 2	—	—
Micrococcus varians	—	—
Micrococcus flavus	+ (6)	+ (3)
Micrococcus aureus 3	+ (5 à 10)	+ (3)
,, ,, 4	+ (5 à 12)	+ (3)
,, ,, 5	+ (5 à 8)	+ (1)
,, ,, 6	+ (5)	+ (2)
,, ,, 7	+ (21)	+ (3)
,, ,, 8	+ (8 à 12)	+ (1)
Streptococcus lactis	—	—
Streptococcus cremoris	—	—
Streptococcus amylolactis	—	—
Sarcina flava	—	+ (3) cf. text
Pseudomonas fluorescens 1	+ (4)	+ (1)
,, ,, 2	+ (6)	+ (1)
,, ,, 3	+ (10)	+ (1)
,, ,, 4	+ (2)	+ (1)
,, ,, 5	+ (6)	+ (1)
,, ,, 6	+ (6)	+ (1)
Pseudomonas putida	—	—
Pseudomonas pyocyanea	+ (4)	+ (1)
Pseudomonas punctata 1	+ (28)	+ (2)
,, ,, 2	+ (14)	+ (1)
Escherichia coli	—	—
Serratia marcescens	+ (8)	+ (1)
Serratia plymouthensis	+ (1)	+ (1)
Bacillus subtilis	—	—
Bacillus licheniformis	+ (8)	+ (3 à 5)

The figures between brackets indicate the number of days after which a positive result could be observed.

Table 1 shows that as a rule there is an excellent agreement between the results of the two methods. There are only two exceptions, *viz.*, *Micrococcus albus* strain 2 and *Sarcina flava*. The latter never showed a positive Eykman test, whilst sometimes, but not regularly, a weak positive result was obtained in the spray test. No explanation for this capricious behaviour can be offered, although it was found that an addition of some calcium chloride to the medium had a favourable effect. Leaving apart this special case which needs further investigation, there are no further indications that with certain bacteria lipase production only occurs after direct contact between bacteria and oil.

As for *Micrococcus albus* 2 we meet with the reverse situation. Here the spray test never yielded a positive result, whilst with the Eykman method after about five days an at first sight convincing white spot in the tallow layer had appeared. The question arose whether this difference in behaviour could be due to the difference in lipolysis substrate used in the two cases: olive oil in the spray test, tallow in the Eykman test. In order to investigate this possibility plates inoculated with various bacteria were sprayed with beef tallow. This could be effected by spraying molten tallow with air which was heated at a suitable temperature, or by spraying a solution of the fat in carbon tetrachloride. The sprayed fat appeared on the plate as more or less irregular, fairly translucent

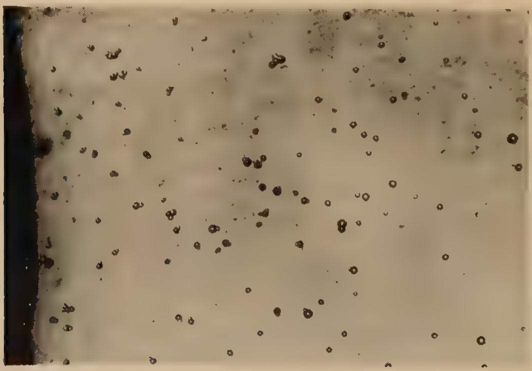

Fig. 4. Action of *Micrococcus albus* strain 2 on beef tallow. The small clumps of fat near the colony (left hand side) have been separated into an opaque solid phase and a transparent oil droplet (30 ×).

clumps from the same dimensions as the oil droplets on normal oil plates. After a few days of incubation a very peculiar phenomenon could be observed. Around colonies of *Micrococcus albus* 2 the fat clumps had become much less translucent than before, whilst attached to the clumps one or a few small clear droplets had appeared (see Fig. 4). Organisms which were positive in the olive oil spray test behaved as might be expected: the translucent fat clumps became granular and opaque. Apart from *Micrococcus albus* 2 all organisms negative in the olive oil spray test did not cause any change of the fat.

In the meantime we had made the observation that T-pol (a 20% solution of some secondary alkyl sulphate, in common use as a detergent) caused white spots on EYKMAN plates. We, therefore, examined the effect of T-pol on plates sprayed with tallow. After streaking sprayed plates with a needle wetted with T-pol, an immediate change of the fat was observable under the microscope. This change was entirely identical with the change caused by *Micrococcus albus* 2. Since T-pol does not cause hydrolysis of fats, in any case not at 25°C., we have to conclude that *Micrococcus albus* 2 produces some compound which like T-pol brings about a separation of the fat into a liquid and a solid phase. It can scarcely be doubted that the pseudo-EYKMAN effect caused by *Micrococcus albus* 2 is due to the excretion of a surface tension lowering substance into the medium ¹).

Leaving apart the two exceptions discussed, both methods yield an identical result. But the figures given in Table 1 make it at once clear that the spray test offers the great advantage that it allows a much quicker decision. Several bacteria which only yielded an often very faint positive EYKMAN test after a considerable lapse of time could be characterized as being lipolytic in the spray test after a few days. Typical examples are the various strains of *Pseudomonas punctata* and of *Micrococcus aureus*.

4. SOME OBSERVATIONS ON CONDITIONS DETERMINING LIPOLYSIS.

In the preceding chapter only bacterial strains have been sub-

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¹) Further investigation has shown that the described pseudo-EYKMAN effect is not obtained with all tallow samples.

mitted to the tests. Moreover, in all cases use has been made of peptone agar as a nutrient medium which implies that the development of the bacteria will have led to a slightly alkaline reaction. Since most investigators indicate that bacterial lipase is most active at a pH of 7.0 or higher, the test conditions used may be deemed to have been favourable.

It seemed desirable to extend the tests to micro-organisms which show optimal development in a more or less acid medium, such as moulds, yeasts etc. We have in this connexion tested a *Penicillium* species and several strains of the yeast *Candida lipolytica*; for all these cultures lipolytic activity had already been established earlier. It was soon found that on using malt agar (pH = 5.2) both EYKMAN and spray tests yielded either negative or only doubtfully positive results. On the other hand growth of these organisms on plain peptone agar was rather meagre, although usually sufficient to give positive indications for lipolysis. If, however, the same strains were investigated on peptone agar to which 0.1% of glucose had been added, growth of the organisms was markedly better, and now in all cases a rapid, distinctly positive reaction occurred in both the EYKMAN and the spray test.

These observations show clearly that in all tests for lipolytic activity due consideration should be given to the cultural conditions under which the organism to be tested develops. On the whole it seems recommendable to perform the lipolytic test in the neighbourhood of pH = 7, but it is dangerous to make this a strict rule, since *e.g.* KIRSH (1935) reports that the lipases present in extracts of *Penicillium oxalicum* and *Aspergillus flavus* had a pH optimum at 5.0.

Another striking example of the influence of external conditions on the result of a lipolytic test was encountered when examining a strain of *Pseudomonas fluorescens*. Although this organism showed good growth both at 25°C. and at 30°C., lipolytic activity as judged by both tests was abundant at the lower temperature and completely absent at 30°C. That the inactivity at the latter temperature is not due to an extreme thermolability of the lipase was shown in a separate experiment. If the bacteria were grown at 25°C., for a few days and the plates subsequently incubated at 30°C. for a week or longer, the lipolytic activity could still be demonstrated at the end of this period by applying the spray test; a positive reaction was obtained within one hour. We must, therefore, conclude

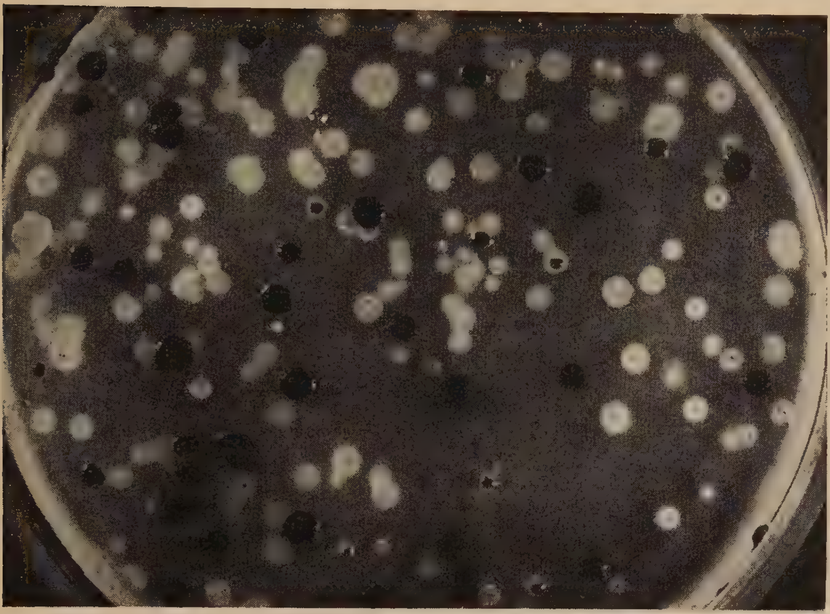

Fig. 5. *Serratia marcescens* strain 2 suspended in the agar of an Eykman plate. Large surface colonies non-lipolytic; small colonies in the agar (barely visible on the photograph) strongly lipolytic.

that the ability of the bacterium to produce lipase is completely suppressed by growth at a temperature of 30°C.

Finally we wish to draw the attention to an observation made by Miss H. G. Numans in the Delft Laboratory already some 20 years ago. As a rule various strains of *Serratia marcescens* are active fat splitters. Miss Numans came across a strain which seemed to make an exception to this rule: streak cultures on an Eykman plate incubated at 30°C. yielded a fully negative result. Since at that time it was suspected that this inactivity might be due to a lack of contact between the bacteria and the fat layer, the experiment was repeated in such a way that a bacterial suspension was mixed with the melted nutrient agar immediately before pouring the agar on the fat layer. The result of such an experiment is rather surprising, as can be seen in Fig. 5.

It is clear that all colonies, with the exception of the large surface colonies, have produced a white chalky field in the more or less transparent fat layer. There can be no doubt that all these colonies, whether they are in contact with the fat or not, are strongly lipolytic, this in contrast to what holds for the much better developed surface colonies. Obviously the low oxygen tension in the agar layer which strongly reduces the growth of the organism, remarkably gives rise to a lipase production. Here too it was observed that, if the bacterium was cultivated at the temperature of 20°C. or lower, the surface colonies too gave a positive EYKMAN test. Thus here again a high temperature seems to be unfavourable for lipase production, but cultivation under reduced oxygen tension seems to reverse this effect.

Summary.

A new method for the detection of the lipolytic activity of microorganisms has been described. In this method small droplets of a fatty oil are sprayed on agar media immediately after their inoculation with the organisms to be tested. In case of lipolytic activity the clear droplets soon become granular and opaque, a change which can easily be established with a low magnification microscope.

The method offers the advantage of direct contact between the growing organism and the oil.

On comparing this spray test with EYKMAN's well-known method for some 20 bacterial strains on the whole identical results were obtained. In one case occasionally a positive spray test was obtained, although the results of the EYKMAN test were negative throughout. In a second case, in which the spray test was negative, it could be shown that the apparently positive EYKMAN test was not due to lipolysis.

In a final chapter some examples were given which clearly demonstrate that lipolytic activity can be largely dependent on external conditions, such as pH, temperature and oxygen tension.

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SPONTANEOUS TUBERCULOSIS IN THE GUINEA PIG

by

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(Received March 28, 1955).

In the annual report 1952 of the National Institute of Public Health [1] HYLKEMA reported that he had found 37 cases of spontaneous tuberculosis in 18600 sections of guinea pigs during the period Sept. 1949—Sept. 1952. These 37 animals all showed a primary localisation of the tuberculous lesions in the lungs and bronchial lymph nodes.

In consequence of these findings the question arose whether the the guinea pigs were either already infected when received at the Institute, or whether they were infected during their stay in our stables. In order to solve this question the following investigations have been made.

Every week ten guinea pigs supplied by various breeders were intradermally injected with human and bovine tuberculin immediately after arrival. Next day the reactions were controlled and the animals sacrificed. One bronchial lymph node of each guinea pig was, after fixation in formalin, microscopically examined for tuberculous lesions. The remaining bronchial lymph nodes were grinded with saline and after treatment with N sulfuric acid inoculated on two Loewenstein tubes one with, and one without glycerol. In accordance with these methods 210 guinea pigs received from 9 breeders were examined. The tuberculin reactions were negative in all the animals. Although in some animals a small enlargement of the bronchial lymphnodes was observed, no tuberculous lesions could be found histologically. Acid-fast bacteria were not cultivated from the lymphnodes of these 210 guinea pigs.

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[1] Verslagen en Mededelingen betreffende de Volksgezondheid Sept./Oct. 1953, p. 167.

It became obvious in the course of the above-mentioned tests that a positive result would probably not be obtained. Therefore a second group of animals (90), supplied by the same breeders as the first group was investigated in the following manner. These guinea pigs were tuberculin tested immediately after arrival and were lodged separately in pots during 2—3 months. After this period the animals were tuberculin tested again, sacrificed, and examined histologically and bacteriologically.

The first tuberculin test was negative in these 90 guinea pigs supplied by 5 breeders. The second test was positive in one guinea pig (261), which had been in our stable for three months; in the other animals the 2nd tuberculin test was negative.

Gross tuberculous lesions were observed in guinea pig 261 as well as in guinea pig 241, which had also been in our stable for 3 months. The pathological-anatomical and bacteriological examinations of the remaining 88 animals were negative.

Guinea pig 241. Bronchial lymphnodes much enlarged (3 × 1 cm) with extensive central caseation. In the cardial lobe of the left lung a sharply circumscribed white tubercle about the size of a matchhead. In the remaining tissue of the lungs some white nodules about the size of a pin-head. Spleen somewhat enlarged with a granular surface caused by pin-head gray nodules. Submaxillar lymphnodes enlarged and centrally caseated. Portal, left inguinal and preascapular lymphnodes somewhat enlarged. Microscopically tuberculous lesions were found in the above-mentioned organs as well as in the macroscopically unaffected liver. A few acid-fast bacteria were present in the lesions. The Loewenstein tubes inoculated with material of this animal showed a dry growth of acid-fast bacteria after an incubation of 4 weeks.

Three guinea pigs inoculated with material of animal 241 died of general tuberculosis after 2 months; after 8 months no tuberculous lesions could be observed in a rabbit injected with this material.

From these results in can be concluded that the lesions in guinea pig 241 were caused by the human variant of *M. tuberculosis.*

Guinea pig 261. Bronchial lymphnodes almost the size of a pigeon egg with central caseation. In the cardial lobe of the left lung a sharply circumscribed gray-white nodule, the size of a pea. Spleen enlarged with many gray-white nodules. Liver somewhat enlarged with granular surface and many sharply defined infarctlike yellow-white areas. Portal, subiliacal and some cervical lymphnodes enlarged with central caseation. Microscopically the lesions were of tuberculous nature and contained few acid-fast bacteria. Loewenstein tubes inoculated with the tuberculous material showed a diffuse, somewhat moist growth of acid-fast bacteria, after 6 weeks of incubation.

Guinea pigs inoculated with a suspension of the bronchial lymphnodes and the liver died of an extensive general tuberculosis after about 10 weeks; a rabbit injected with this suspension died of general tuberculosis after 3 months (regional lymphnodes, lungs, spleen and kidneys).

From these results it may be concluded that the tuberculous process in this guinea pig was caused by the bovine type of *M. tuberculosis.*

The most striking features in both guinea pigs supplied by two breeders were the much enlarged bronchial lymphnodes, in combination with a large caseated nodule in the lungs (primary complex). It may be assumed from the localisation of this primary complex that the infection occurred via the tractus respiratorius.

The fact that in the first group of 210 animals, examined immediately after arrival, no tuberculous lesions were found, whilst 2 of 90 guinea pigs which were kept in our stables for 3 months showed tuberculous lesions, indicate that infection probably took place during their stay at our Institute.

The source of the infection has not been found.

Summary.

Description of 2 cases of spontaneous tuberculosis in guinea pigs. It is assumed that infection took place during their stay at the Institute. The source of infection has not been found.

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